THE UPTAKE OF FERULIC ACID IN RUMINANTS AND THE ANTIOXIDANT CAPACITY OF MILK AND MILK REPLACERS

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THE UPTAKE OF FERULIC ACID IN RUMINANTS AND THE ANTIOXIDANT CAPACITY OF MILK AND MILK REPLACERS

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Ferulic acid (FA) is a phenolic compound with antioxidant and anti-cancer properties that naturally occurs in forages. Bound FA is not typically available for ruminant absorption and is excreted primarily in the feces as a component of lignin. However, FA can be released from the lignin complex to increase digestibility in vitro using enzymatic pretreatments. The first objective of this research was to investigate the likelihood of free FA uptake in sheep and the impact of increasing dosages of free FA on DMI and rumen microbes in vitro. There were no negative repercussions on microbial in vitro digestion of alfalfa NDF or on lamb DMI as a result of FA administration. Moreover, the dose dependent presence of FA in the urine of lambs confirmed FA uptake in the blood. Thus, the second objective was to determine if orally dosed free FA could be transferred into the milk of lactating cows. It was observed that free FA could be transferred into milk, and is also present at basal levels of 4.1 µg/L in cows fed maize silage. Given the peak concentration of FA in bovine milk of cows receiving 121% of the FA present in their diet (1500 µg/L), the amount of FA that could potentially be released from feeds using enzymatic pretreatments would still be far below the reported sensory detection threshold of 62 mg FA/L. The primary benefit of increased FA in milk is its effects on antioxidant activity (AOA). However, the benefit of studying one compound's contribution to total AOA in milk is limited due to the potential effects of many compounds and their synergies. Therefore, the final objective was to describe the AOA of bovine milk, compared to six calf milk

replacers (CMR), varying in fat and protein. Although 69% of calves in the U.S. are raised on CMR, CMR are not formulated for AOA. Five of the 6 CMR analyzed were significantly lower in AOA than milk (52.7 µmol vitamin C equivalent/mL). This research revealed opportunity to better meet the needs of calves in a critical stage of life when AO can enhance immune defense.

BIOGRAPHICAL SKETCH

Melanie Amanda Soberon was born on May 12, 1983 in Peoria, Illinois to Stephen and Tamia Schotthofer. She and her sisters, Melissa and Stephanie, grew up on a mini-farm, surrounded by horses, sheep, chickens, dogs and cats; summertime was filled with putting up hay, selling the family's garden produce on her grandma's gift shop porch and preparing for the 4-H fair. At the age of 15, her family relocated to Cochrane, Wisconsin to pursue their dream of dairy farming full-time. Due to poor milk prices at the time, the family altered their goals to dairy sheep production, and Melanie worked alongside her parents and sisters to expand a flock of 37 meat sheep into a 450+ ewe flock with dairy sheep genetics. On weekends, she helped the family direct market the lamb produced at the St. Paul Farmer's Market. Though she became proficient at working with sheep, her interests remained in the sphere of dairying, whether with sheep or cows. After finishing high school at Cochrane-Fountain City in 2001, she attended the University of Wisconsin-Madison on scholarship. While there, she developed a love for science writing. In 2005, she graduated from UW-Madison with a double major in Animal Sciences and Life Science Communications, with emphases in Business. Melanie came to Cornell University to earn a Master of Science degree in Sheep Nutrition under the direction of Dr. Michael Thonney. In August of 2007, she then began a PhD with Dr. Debbie Cherney in the area of Ruminant Nutrition, incorporating aspects of Food Science to produce the research in this dissertation. Throughout her studies, Melanie discovered a joy in teaching animal science, which led her to participate in the Cornell Center for Teaching Excellence's Future Faculty Teaching Certificate program. This culminated in her first publication in the scholarship of teaching and learning, also included in this dissertation. In June of 2007, she married Fernando Soberon and they were blessed with two children, Fernando Stephen in March 2009, and Elena Grace in November 2011.

To my family:

Fernando

Fernando Stephen and Elena Grace

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Within Morrison Hall, there is a second family of people who contributed to my development as a researcher, teacher and scientist. I have been blessed to have in my committee chair, Debbie Cherney, a warm and compassionate person, who has guided me throughout the dissertation, while offering many opportunities for my own independent ideas and growth. Each of my committee members has contributed uniquely to my growth and development. The mentorship I received from Dave Barbano, especially in the grant writing process of his course, was key in the development of my scientific writing and experimental design, and his consultation during different phases of the milk analyses was most helpful. Jerry Cherney was a patient and thorough teacher of the many forage lab techniques that I implemented in his laboratory, and his editing of my first manuscripts was instrumental as I learned how to write for scientific publications. The example that Tom Overton has given has

been a constant inspiration to me of how a healthy balance of extension, research and teaching can be achieved, and he was generous in the usage of his laboratory for my milk analyses.

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Due to the collegial nature of this department, it is difficult to thank only a few when in fact, the department as a whole, contributed to my development as an individual, as a teacher and as a researcher. Dan Brown gave me an incredible opportunity when he offered to first let me co-instruct and then instruct solely the Animal Science 1120 Sustainable Animal Husbandry summer course. He will always have my deep gratitude for the way he saw my interest and generously provided opportunity to develop it. Deloris Bevins has made it a pleasure to do any business in the main office and I will miss our regular interactions, her professionalism and friendship. Susanne Pelton and Debbie Ross have been constant sources of support and expertise during my analytical journey. There are many others whom I have enjoyed working with in various capacities and whom I also consider to be friends

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LIST OF ABBREVIATIONS

ADG	average daily gain
AO	antioxidant
BW	body weight
CMR	calf milk replacer
CP	crude protein
DMI	dry matter intake
FA	ferulic acid
NE	net energy
NDF	neutral detergent fiber

CHAPTER 1

LITERATURE REVIEW

Introduction

While milk is already considered a source of nearly complete nutrition, opportunities exist to further boost its nutritive value, sensory characteristics, processing and storage stability. Diet-derived phenolic compounds influenced the odor-active and ultraviolet-absorbing compounds in milk of lactating cows, yielding potential sensory and health impacts for the milk produced (Carpino et al., 2003; Besle et al., 2010). The selection of dietary ingredients to include in the diets of lactating cows with the purpose of improving the quality of the milk is an area of study requiring the cooperation of multiple disciplines of study, including crop science, ruminant microbiology and nutrition, as well as food science. The influence of diet-derived compounds upon milk quality and sensory characteristics in lactating ruminants is dependent upon ruminal digestion, the possible transformations that may occur, the absorption of compounds into the circulatory system, the mammary uptake of these compounds as well as the storage and processing of the milk.

One compound detected by gas chromatograph olfactometry in Ragusano cheese was vanillin, thought to be derived from ferulic acid (FA) (Carpino et al., 2003). The authors speculated about the origin and implications of FA in milk, given some of its unique properties (personal communication) and health-promoting effects.

The compound

Ferulic acid, or 4-hydroxy-3-methoxycinnamic acid is a low molecular weight compound (194.2), soluble in alcohol as well as water at 5,970 mg/L at 25°C. Historically, FA has been used as an effective component of Chinese medicinal herbs due to its health benefits; today, it is attributed with a variety of physiological

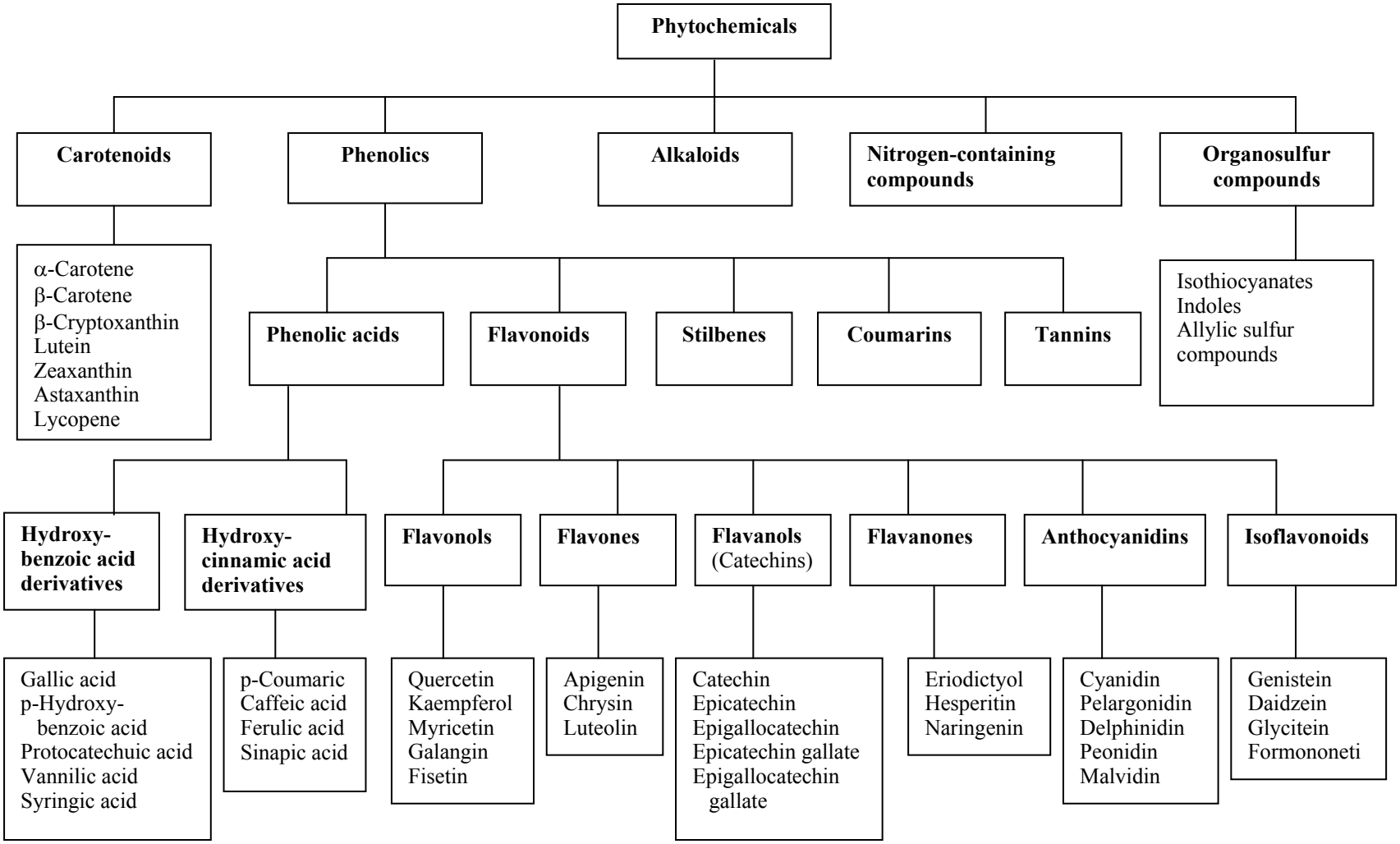

Figure 1.1 Classification of dietary phytochemicals (Liu, 2004).

functions including antioxidant, antimicrobial, anti-inflammatory, anti-thrombosis, and anti-cancer activities (Ou and Kwok, 2004).

Phytochemicals can be classified according to their structure and function; one subcategory of phytochemical includes phenolic acids. Phenolic acids represent a subgroup of secondary metabolites found in plants during normal development or in response to stress events such as plant defense against UV radiation or pathogens (Naczk and Shahidi, 2006). Phenolic acids can then be categorized as either hydroxybenzoic or hydroxycinnamic acids, of which FA is considered a hydroxycinnamic acid (Figure 1.1). Ferulic acid is usually present as a simple ester with quinic acid or glucose, polysaccharides or some carboxylic acids (Mattila and Kumpulainen, 2002).

Origin and occurrence of ferulic acid

Ferulic acid is a ubiquitous plant component that arises from the metabolism of phenylalanine and tyrosine via the shikimic acid pathway (Jung and Fahey, 1983a; Srinivasan et al., 2007). In fact, the shikimic acid pathway is attributed with the synthesis of all phenolic lignin precursors (Van Soest, 1994). Ferulic and *p*-coumaric acids are the major phenolic acids of plant cell walls, where they may be linked to pectins, sugars, polysaccharides, lignins, amines, long-chain alcohols, glycerol or cross-linked to cell wall polysaccharides in the form of dimers (Nazck and Shahidi, 2006). Ferulic acid can be present in free or bound forms; it tends to occur in free forms in seeds and leaves, but in a bound form within the lignin portion of plants (Srinivasan et al., 2007). In barley seeds, buckwheat grits and barley bran, FA is the predominant free phenolic acid (Nazck and Shahidi, 2006). In common flour and wheat kernels, FA accounts for 90% of the phenolic acids present, and it is a major constituent of fruits like oranges and tomatoes as well as vegetables like asparagus and

sweet corn (Srinivasan et al., 2007). Ferulic acid has also been identified in blueberries, potatoes, apples, carrots and a variety of beers (Mattila and Kumpulainen, 2002; Coghe et al., 2004; Nazck and Shahidi, 2006).

Within ruminant feeds, ferulic and *p*-coumaric acids are the two most abundant phenolics in forages, and lignin in the cell wall is the largest source of phenolic material in the plant (Jung and Fahey, 1983a). Chesson et al. (1982) reported FA to be 0.25% and *p*-coumaric acid to be 0.23% of the dry weight of grasses analyzed. As forage plants mature, free monomeric ferulic and *p*-coumaric acids become incorporated into the lignin complex to provide structural support for the plant. Lignin is a heterogenous biopolymer that is a component of plant cell walls, along with cellulose, hemicellulose and pectin (Van Soest, 1994). Lignin covalently links to hemicellulose, and in the process, cross-links with polysaccharides (Nazck and Shahidi, 2006). These aspects of its structure are key to its role in the plant of conferring mechanical strength and aiding in water transport.

During the polymerization process of lignin, an almost random series of bonding ensues, resulting in lignin's complex structure, (Jung and Fahey, 1983a). It appears that *p*-coumaric and ferulic acids use their functional hydroxyl and carboxyl groups to act as cross linkages between lignin and nonstructural carbohydrates (Jung and Fahey, 1983a). The carbon-carbon and ether linkages of lignin are resistant to hydrolysis (Jung and Fahey, 1983a), but the ester linkages are cleavable by microbial esterases. Thus, the subcomponents of lignin are often classified as core and non-core lignin fractions, where core lignin refers to the highly condensed phenylpropanoid matrix of lignin that is generally considered indigestible, and non-core lignin refers to the extractable phenolics, namely *p*-coumaric and ferulic acids (Jung and Fahey, 1983a; Van Soest, 1994).

Interest has been shown in identifying a consistent relationship between

phenolic acid content of a forage and some aspect of NDF digestibility in hopes of using a forage's phenolic acid content to predict NDF digestibility and provide information about the cross-linking of lignin and carbohydrates (Dann et al., 2006).

Release of ferulic acid from lignin complex

In human nutrition, researchers have investigated the release of FA to improve its bioavailability in human diets (Bourne and Rice-Evans, 1998; Adam et al., 2002). In a typical ruminant diet, a significant quantity of FA is present, albeit linked within the lignin complex of plants. Cherney et al. (1989) reported that treatment of grass forage crops with NaOH increases digestibility. The same effect would not be expected in alfalfa forage crops because there are very few ester linkages of FA and *p*-coumaric acid to the cell wall in legumes that would be susceptible to the base treatment (Cherney et al., 1989). While the linkage between FA and lignin can be either an ether or ester bond, FA is covalently linked to polysaccharides by ester bonds (Mathew and Abraham, 2004). It was hypothesized that the ester linkage between FA and its attached sugar of the cell wall feruloyl-polysaccharide may be cleaved by specific enzymes (Yu et al., 2005). This cleavage would expose potentially digestible carbohydrates that were previously trapped within the lignin complex to rumen microbial digestion. Methods to free FA from the lignin in feed would therefore increase feed digestibility and reduce feed expenses for dairy producers.

It is worth noting that while rumen microbiota such as *Fibrobacter succinogenes* and *Butrivibrio fibrosolvens* have feruloyl esterases that could potentially cleave accessible ester linkages (Besle et al., 1995), this process occurs inefficiently in vivo and it is unclear why they do not appear to cleave a quantitative number of ester linkages (Dann et al., 2006). Some point to the supposed inability of rumen microbiota to express the appropriate ensemble of enzymes as an explanation

for less than maximum fiber digestion (Krause et al., 2003). Following the assumption that rumen microbiota may simply be unequipped with appropriate fibrolytic capabilities, a new interest in genetically modified rumen bacteria has emerged as the focus of intensive research (Krause et al., 2003).

Other active areas of research have discovered that the activity of feruloyl esterases can be greatly enhanced by the addition of xylanases, pectinases, cellulases and other cell wall degrading enzymes (Mathew and Abraham, 2004). Therefore, various researchers have experimented with multienzymatic combinations in vitro and in incubations with a variety of feedstuffs to release FA from lignin and increase digestibility of that feedstuff.

Ninety-five percent of the available FA from wheat bran was released after 5 h incubation with FA esterase-III and xylanase (Faulds and Williamson, 1995). In brewer's spent grain, 65% of the available FA was released after 3 h incubation with a preparation called Ultraflo that included β-glucanase, cellulose, xylanase, pentosanase and arabinase (Faulds et al., 2002). Yu et al. (2002) used FA esterase and xylanase to release 69% of the available FA in oat hulls after incubation. In a later study, they observed increases in the in vitro dry matter disappearance of oat hulls (12.6% increase), alfalfa hay (2.3% increase) and wheat straw (5.1% increase) using an enzymatic treatment of FA esterase, xylanase, cellulose, endo-glucanase and β-glucanase (Yu et al., 2005). The substantial increase seen in oat hull digestibility as well as wheat straw digestibility with the multienzymatic treatment of Yu et al. (2005) was not observed in alfalfa digestibility. This may have been due to the decreased amount of *p*-coumaric and ferulic acids in comparison to oat hulls as well as the fact that legume lignins are more condensed and potentially less reactive than grass lignins (Jung et al., 1983b). The preliminary findings of these lines of research using multienzymatic pretreatment of poorly digestible feeds suggest positive effects on

increased digestibility and therefore, increased feed efficiency. Commercial forms of these enzymatic pretreatments however, are not yet widely available.

Inhibitory effects of bound versus free FA in the rumen

There are examples of phenolics in nature that suggest intrinsic characteristics of phenolic acids which may be inhibitory to microorganisms. For instance, it is postulated that the natural durability of wood may be a result of the potential toxicity to microorganisms of phenolics deposited in the process of heartwood formation (Jung and Fahey, 1983a). In addition, phenolic compounds have historically been used as food preservatives due to their inhibition of microbial growth (Jung and Fahey, 1983a).

In the rumen, a large proportion of ingested lignin is converted to a soluble lignin-carbohydrate complex that is approximately 20% carbohydrate and contains the same elements combined in the same proportion but with different molecular weights (Jung and Fahey, 1983a). These phenolic-carbohydrate complexes have been shown to suppress rumen bacterial growth (Akin et al., 1988; Griggs et al., 1989) and microbial digestion in multiple ways, one of which may be binding with enzymes and forming nutritionally unavailable polymers with dietary proteins (Jung et al., 1983b). However, Jung et al. (1983b) could not conclude if the detrimental effects of simple phenolic monomers on digestion and metabolism of animals were due primarily to interactions with nutrient digestion or disruption of metabolism. Another manner of inhibition is the physical barrier to microorganisms of the cross-linking of cell walls with phenolic acids (Nazck and Shahidi, 2006); this can pose a challenge to rumen microbial species in their mission to access digestible nutrients. Likely due to this mechanism, phenolic-carbohydrate complexes have been shown to inhibit the in vitro fermentation of structural carbohydrates (Chaves, 1982; Jung, 1988; Cherney et al.,

1993) by reducing the rate of polysaccharide digestion (Jung and Allen, 1995). Trans-*p*-coumaric acid is consistently identified as the most effective inhibitor of microbial forage digestion (Herald and Davidson, 1983; Akin et al., 1988). However, while *p*-coumaric acid esters are more inhibitory to cell wall digestion than ferulate esters, the majority of *p*-coumaric acid is esterified to lignin, and according to Jung and Allen (1995), less likely to cause a direct effect on polysaccharide digestion. Ferulic acid esters of arabinoxylan can inhibit digestion by hindering the alignment of xylanase with its substrate, thus preventing hydrolysis of the attached polysaccharide (Jung and Allen, 1995).

It is less clear how free phenolic monomers, such as free FA, are inhibitory to microbial digestion (Cherney et al., 1989). Despite the reported antibacterial and antifungal properties of free FA (Barber et al., 2000), there is evidence that rumen microbes can adapt to increased concentrations of free FA in vitro (Cherney et al., 1993). Moreover, the inhibitory activity of free FA will vary based upon factors such as the bacterial species, the plant species and the pH of the environment (Herald and Davidson, 1983; Cherney et al., 1989). In a study of the effects of different concentrations of FA on cellulolytic bacteria, when concentrations of FA that might be ingested were added to rumen bacteria, three strains of cellulolytic bacteria were affected; their growth was retarded, but not actually suppressed (Chesson et al., 1982). All three strains also exhibited at least a limited ability to modify FA to less toxic forms (Chesson et al., 1982).

Ferulic acid is one of about 25 phenolics considered to be universal in plant-derived diets (Singleton and Kratzer, 1969). The question remains whether there would be appreciable negative physiological activity associated with the amount present in a normal plant diet if increased FA was released from the plant cell wall (Singleton and Kratzer, 1969; Chesson et al., 1982; Barber et al., 2000).

Fate of ferulic acid in the rumen

Jung et al. (1983b) identified five phenolic monomers in the rumen fluid of sheep fed alfalfa hay, smooth bromegrass hay, corn stalklage and soybean stover. Ferulic acid was not included among them, likely because it was present primarily in a bound form in the diet. When discussing the digestive fate of FA, it is important to note the distinction between what is known about the fate of bound versus free forms of FA. Allinson and Osbourn (1970) observed that simple, nonconjugated phenolic units of the lignin fraction, such as free FA, were digested or modified in the rumen by unknown mechanisms while those phenols that were conjugated appeared unaffected. As aforementioned, there are also many examples of inhibitory effects of bound FA upon microbial digestion. However, once FA is released in the rumen, there are a number of possible outcomes for free FA monomers. In the rumen, reduction, demethylation, dehydroxylation and decarboxylation reactions result in several absorbable aromatic compounds (Besle et al., 1995). They could be taken up across the rumen wall, degraded or transformed in the rumen, or could pass out of the rumen undigested. Besle et al. (1995) diagrams a speculative but fairly comprehensive attempt of the reactions which may occur to free FA in the rumen given sufficient bacterial populations and time (Figure 1.2). In his review on page 38, he describes the possible reactions in Figure 1.2 below:

> "In this ecosystem, ferulic acid is hydrogenated by several cellulolytic bacteria and by Wolinella succinogenes…The product formed, 3-methoxyphloretic acid, may then be slowly decarboxylated or demethylated by rumen anaerobes to yield, after 7 days incubation, 4-hydroxyphenylacetic acid, vanillic acid and then probably acetate, isobutyrate plus n-butyrate. Vanillic acid also has been observed in the cecum of rats fed 2-14C ferulic acid. In the rumen, it is probably easily converted to catechol but catechol seems resistant and will take longer for degradation – more than 3 weeks - and may previously be converted to phenol. The direct decarboxylation of ferulic acid into 4-vinylguaiacol has been observed in the gut of monogastrics and under other anaerobic conditions. Ferulic acid is also demethylated to caffeic acid and then dehydroxylated to yield *p*-coumaric acid."

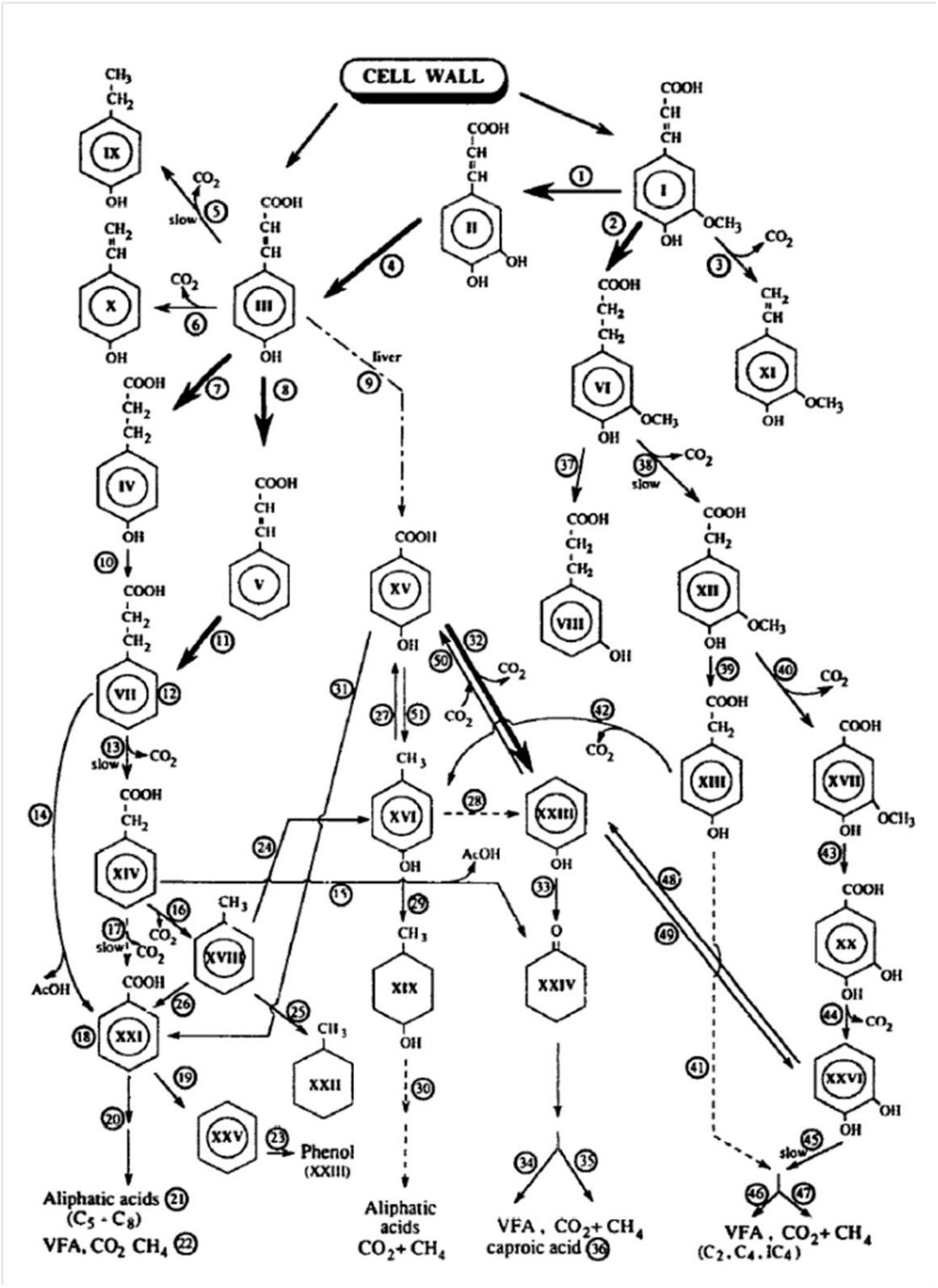

Figure 1.2 Compounds which may result from the degradation of ferulic and *p*-coumaric acids in the rumen and possible pathways. (Besle et al., 1995; Figure 2)

I, Ferulic acid; II, caffeic acid; III, *p*-coumaric acid; IV, phloretic acid; V, cinnamic acid; VI, 3-methoxyphloretic acid; VII, 3-phenylpropionic acid; VIII, 3-hydroxyphenylpropionic acid; IX, 4-ethylphenol; X, 4-vinylphenol; XI, 4-vinylguaiacol; XII, homovanillic acid; XIII, 4-hydroxyphenylacetic acid; XIV, phenylacetic acid; XV, 4-hydroxybenzoic acid; XVI, *p*-cresol; XVII, vanillic acid; XVIII, toluene; XIX, 4-methylcyclohexanol; XX, gallic acid; XXI, benzoic acid; XXII, methylcyclohexane; XXIII, phenol; XXIV, cyclohexanone; XXV, benzene; XXVI, catechol. (1) MC, E. cloaca, E. cloaca, (2) rumen cellulolytic bacteria, W. succinogenes, (3) Aerobacter, Bacillus, (4) MC, E. cloaca, E. cloaca,(5) rumen, rat DT, (6) Aerobocter, Bacillus, rat DT, (7) rumen cellulolytic bacteria, rumen, rat DT, (8) E. cloaca, rumen, rumen, MC, (9) urine of sheep, rat liver, (10) rumen, (11) MC, MC, E. cloaca, (12) Compound observed by rumen, MC (13) MC + BESA, MC, E. cloaca, MC, (14) MC + BESA, MC rumen or sewage sludge, (15) MC, MC rumen or sewage sludge, (16) MC + BESA, MC, (17) Not demonstrated reaction, (18) Compound observed in urine of sheep, E. cloaca, rumen, MC, (19) MC + BESA, MC, (20) MC, MbC, (21) E. cloaca, (22) Products detected in bacterium + methanogen, MC (23) MC, MC + BESA, (25) MC + BESA, MC, (26) MC + BESA, MC, (27) P. putida, MC, bacterium PC07 + nitrate, (28) MC, (29) MC + BESA, MC, (30) MC + BESA, MC, (31) MC, Pseudomonas K 172 + nitrate, (32) rumen, urine of sheep, MC rumen or sewage sludge, (33) MC, MbC, (34) MC, (35) MC, (36) MbC, (37) S. sucromutans, E. limosum, A. woodii, (38) rumen, (39) rumen, (40) rumen, rat DT, (41) rumen, (42) rumen, (43) A. woodii, MC, S. sucromutans, E. limosum, (44) MC, MbC, (45) rumen, MC, (46) rumen, (47) MC, (48) MC + BESA, (49) MC + BESA, (50) Pseudomonas K 172 + nitrate, MC, (51) rumen. Solid bold arrow, major route; solid arrow, minor route; hatched arrow, route not demonstrated; hatched/stippled arrow, reaction occurring in the body; VFA, volatile fatty acids; MC, MbC. methanogenic or microbial consortia from ecosystems other than the rumen; DT, digestive tract; BESA, 2-bromoethanesulfonic acid.

Thus, while monoaromatic compounds like FA are potentially fully degradable in the rumen, the actual degradation of FA in the rumen is limited by the low redox potential, the small populations and slow growth of the microorganisms that use monoaromatics, as well as a limited ruminal retention time (Besle et al., 1995). As Van Soest describes in the Nutritional Ecology of the Ruminant, anaerobic metabolism requires that oxygen be removed from the substrate to produce a reduced product, which explains why dietary substances with low oxygen content like phenolics, fatty acids and waxes are metabolized very slowly or not at all by anaerobes (1994). One might expect these compounds to be degraded to methane in the event

that rumen retention time was sufficiently long, but in any case, phenolics would not be expected to provide energy for fermentation (Van Soest, 1994). Rather, it appears that free FA in the rumen is most commonly reduced to a few common derivatives. The FA derivatives most commonly reported in rumen fluid are phenyl-3-propionic acid at 50 to 80% of rumen aromatic acids detected, phenylacetic acid at 13 to 50% of rumen aromatic acids detected, and cinnamic and benzoic acids at 0 to 7% and <2% of rumen aromatics detected, respectively (Chesson, 1982; Martin, 1982; Besle et al., 1995; Cremin, Jr. et al., 1995). The ruminal degradation of phenyl-3-propionic acid is likely low because decarboxylation occurs preferably on 4-hydroxylated aromatic compounds (Besle et al., 1995). An example of this reported by Besle et al. (1995) is when *Enterobacter* rapidly produced phenyl-3-propionic acid, the decarboxylation into phenylacetic acid occurred at a low rate of 20% in 15 d.

Digestive fate of ferulic acid

Singleton and Kratzer (1969) reported that often 10% and sometimes more than 50% of fed lignin is solubilized and may be absorbed in ruminants and rabbits. Fahey et al. (1980) found that approximately 60 percent of lignin disappearance occurred in the rumen. They speculated that if the major phenolics in alfalfa and bromegrass cell walls (*p*-coumaric acid, ferulic acid, and vanillin) were the structural monomers of lignin, then the apparent digestibility of lignin may be due to their disappearance (Fahey et al., 1980). They also observed a decline in concentration of each of these phenolics after passage through the sheep digestive tract indicating that some absorption and metabolism may occur. Fahey et al. (1980) postulated that the phenolics may bind to nitrogen fragments or other low molecular weight materials and be excreted in the feces.

Evidence also exists in support of lignin metabolism in the lower gut. Allinson

and Osbourn (1970) discovered that after feeding sheep sainfoin, they could identify a chemical feed fraction that was not found in feed or rumen contents but was analyzed quantitatively as lignin and absorbed wavelengths typical of nonconjugated phenols. In humans, esterified hydroxycinnamates are not cleaved in the gastric lumen or the small intestine; rather, they are cleaved by gut microflora esterases in the colon (Rechner et al., 2002). In fact, in humans, over 95% of the total release of feruloyl groups is thought to take place in the colon (Kroon, et al., 1997). Then, they can be absorbed as free *p*-coumaric, ferulic or caffeic acids, or degraded in the colon into dihydroferulic, 3-hydroxy-hippuric or hippuric acids (Rechner et al., 2002).

The metabolism of free phenolic acids in the ruminant is not fully understood. Jung and Fahey (1983a) implicated that phenolic compounds that can form glucuronides in the liver may undergo enterohepatic circulation, be excreted in the bile and then hydrolyzed by intestinal bacteria into free phenolics, capable of reabsorbtion. This process may succeed in releasing free phenolics from bound forms to allow their subsequent absorption. Martin (1982) infused several hydroxycinnamic acids in the rumen of sheep and recovered 60 to 106% of the acids in urine as benzoic acid and 20% as cinnamic acid.

While the digestive fate of free FA in ruminants is largely speculative, a wider range of literature has investigated the bioavailability and metabolism of FA and other polyphenols in monogastrics. After in situ gastric administration of free FA to rats, Zhao et al. (2004) observed 74% disappearance within 25 min; they concluded that free FA could be absorbed at a high absorption rate from the stomach of monogastrics. Although the absorption mechanism was not clearly established, Zhao et al. (2004) hypothesized that FA could diffuse across the stomach mucosa in an undissociated form due to the strong acidic environment of the stomach; there may also be a monocarboxylic acid transporter involved in gastric absorption of FA. They recovered

the FA dosage in the gastric mucosa, portal vein plasma, celiac arterial plasma, bile and urine. The oral dose of FA was recovered in high concentrations in rat plasma as free FA soon after the administration, but was no longer detected 2 h post-administration. Similarly, in an earlier study in rats, the orally administered dose of FA had almost completely disappeared from blood plasma by 30 min post-administration (Zhao et al., 2003). Once present in the artery, FA was primarily in a conjugated form in plasma. Thus, Zhao et al. (2004) concluded that FA is metabolized mainly in the rat liver, and reintroduced into the circulation mainly in conjugated forms. Rechner et al. (2002), who took blood samples 1, 3 and 5 h post-ingestion of polyphenols, observed FA in plasma as a glucuronide. Ferulic acid was excreted in the urine in both free and conjugated forms (Zhao et al., 2004).

Bourne and Rice-Evans (1998) investigated the bioavailability of FA in humans from tomato consumption. They recovered 11 to 25% of the FA ingested in the urine in the forms of free FA and feruloyl glucuronide. Due to its ability to be absorbed intact and its lifetime in the general circulation, they felt that FA had the correct pharmokinetic properties to function physiologically (Bourne and Rice-Evans, 1998).

Using an in vitro assay to study the release of covalently bound FA in the human colon, Kroon et al. (1997) were able to show that significant amounts of esterified feruloyl groups could be solubilized from plant fiber in the large intestine, but the rate of solubilization was dependent upon the plant source. They also confirmed that the free FA released in the colon was different from other dietary phenolics in that it did not return to bind to the residual fiber in the colon. Instead, Kroon et al. (1997) hypothesized that it was either rapidly utilized by gut microorganisms or transformed to other phenolic forms because the levels they observed in their model colon were low and did not reflect the amounts released from

the fiber source. According to Rechner et al. (2002), FA can also be absorbed after its cleavage in the colon.

Rechner et al. (2002) fed human subjects (n=20) polyphenol-rich diets and tracked their blood and urine to monitor the absorbed forms of the polyphenols. For both blood and urine samples, great individual variation was observed in the absorption and metabolism of polyphenols. In a 12 h timeframe, in response to the polyphenol-rich diet, they observed roughly double the urinary excretion of FA as well as isoferulic, sinapic, vanillic, homovanillic, 3-hyroxyhippuric and 4-hydroxyhippuric acids. Hippuric acids were increased more than any other metabolite, in a quantity that exceeded all the other conjugates and metabolites put together, indicating that hippuric acid is the final metabolic fate of most polyphenols (Rechner et al., 2002). Besle et al. (2010) confirmed the presence of increased hippuric acid in milk of cattle fed polyphenol-rich diets as well.

Analyzing ferulic acid in milk

When measuring total phenolic compounds in milk, O'Connell and Fox (2001) reported that phenolic compounds are present in ruminant milk in considerable amounts, measurable by milligrams per kilogram. However, most analytical literature concentrates on phenolic acids from plants and there are very few instances of quantified FA concentrations reported for milk. In fact, Besle et al. (2010) stated that their observed concentrations of FA in milk were the first instances to their knowledge in which FA was identified in bovine milk; their values ranged from 0.9 to 14.7 µg FA/L depending upon diet. One of the reasons FA is rarely reported in milk is the lack of standardized methods for quantifying FA in milk.

The determination of phenolic acids is admittedly complicated due to the different chemical natures and variance in sensitivities to extraction conditions and

hydrolysis (Mattila and Kumpulainen, 2002). Naczk and Shahidi (2006) wrote an informative review on the extraction and analysis of phenolics in different foods. They acknowledge the diversity of phenolics and the complications that can occur when phenolics, such as FA, form complexes with other plant components like carbohydrates or proteins (Naczk and Shahidi, 2006). Therefore, alkaline hydrolysis, often with NaOH, or acid hydrolysis is employed to liberate bound phenolics (Krygier, et al., 1982; Naczk and Shahidi, 2006). Alkaline hydrolysis has been used effectively to release bound phenolics in forages as well as a variety of foods including oranges, grapefruits, wheat bran and sugar beets (Peleg et al., 1991; Kroon, 1997). After alkaline hydrolysis, the sample pH is adjusted to 1.5 to 2.5 prior to extraction. Phenolic extraction is achieved with solvents such as methanol, ethanol, propanol, acetone, ethyl acetate, water, dimethylformamide or mixtures of the aforementioned solvents (Mattila and Kumpulainen, 2002; Naczk and Shahidi, 2006).

Over time, various quantification techniques have been assessed for limitations and practicality of use for phenolic analyses, including high-performance liquid chromatography, thin-layer chromatography, gas-liquid chromatography, gas chromatography-mass spectrometry, and capillary electrophoretic methods (Mattila and Kumpulainen, 2002). While spectrophotometric methods have been used for total phenolics determination, developing a satisfactory UV assay has been a difficult process (Naczk and Shahidi, 2006). This has been in part due to the interference of other UV-absorbing substances like proteins, nucleic acids and amino acids. In addition, the absorption of simple phenolics, commonly between 220 and 280 nm, can be affected by the nature of the solvent used and the pH of the solution (Naczk and Shahidi, 2006). Phenolic compounds are separated, purified and identified using chromatographic techniques such as liquid chromatography, high-performance liquid chromatography and high-speed countercurrent chromatography (Naczk and Shahidi,

2006) but HPLC is presently the most widely used quantification method for phenolic analyses (Mattila and Kumpulainen, 2002).

Of the three publications with reports of FA concentrations in milk, two used HPLC with photo-diode array to identify the FA in human breast milk and bovine milk, respectively (Li et al., 2009; Besle et al., 2010). However, a major problem when analyzing phenolics in dairy products can be the column clogging associated with the abundance of proteins; proteins can also interfere with UV and MS signals of target analytes (Redeuil et al., 2009). Redeuil et al. (2009) also observed an interference of procyanidin B1 with FA when analyzing milk-based products, which resulted in incomplete separation on the UV chromatogram. Therefore, they developed an LC-MS method, designed to be more accurate in determining individual compound quantification for individual phenolic acids and flavan-3-ols in complex food matrices (Redeuil et al., 2009).

Impacts of ferulic acid in milk

Besle et al. (2010) attributed the presence of the majority of ultraviolet-absorbing compounds identified in milk (including FA) to the ruminal degradation of soluble polyphenols and cell wall aromatics. If a sufficient amount of free FA is absorbed and taken up by the mammary gland of lactating cattle, it could potentially influence milk flavor, microbiology, storage and health benefits.

In terms of the influence of FA on milk flavor, it is not likely that increased concentrations of FA in milk will affect milk flavor due to its high detection threshold. Work and Camire (1996) reported the detection threshold of FA to be 62,000 µg/L in deionized water and Meilgaard (1975) reported it to be 600 mg/L in beer. Potential sensory impact of increased FA in milk is more likely to occur over time with storage if FA undergoes direct decarboxylation into 4-vinylguaiacol, which has been observed

in anaerobic conditions (Besle et al., 1995). In citrus juices, increased storage time is associated with both decreased FA and increased 4-vinylguaiacol, which is responsible for detrimental off-flavors in the juices (Naim et al., 1988; Fallico et al., 1996). The flavor of 4-vinylguaiacol, which is described as medicinal, phenolic, clove-like or smoky (Coghe et al., 2004), combined with its low sensory threshold (300 µg/L in beer), would be detrimental in milk. Further research would be required to determine the effects of length of storage and type of pasteurization on milk with increased FA concentrations.

The potential impact of increased FA concentrations in milk is more likely to manifest itself in the cheese made from that milk. Free phenolic compounds may be released through chemical or enzymatic hydrolysis during the maturation of the cheese or pasteurization of the milk (O'Connell and Fox, 2001). This is noteworthy because free phenolics contribute more to flavor than conjugated phenolic compounds, despite the fact that the majority of phenolics in milk occur as conjugated (Lopez and Lindsay, 1993). Sensory impact of free FA in cheese will be dependent upon the type of cheese being made. Indigenous phenolic compounds are those present in milk but derived from pasture, animal metabolism or microbial activity in dairy products. Therefore, indigenous FA may contribute in a desirable manner to a Roquefort cheese with a stronger flavor profile but be found undesirable in a milder tasting cheese like Cheddar (O'Connell and Fox, 2001). Similarly, certain phenolic compounds did not appear in Camembert cheese until it was ripened, indicating that during ripening, transformations may occur that affect the flavor profile or microbiological state of the cheese (O'Connell and Fox, 2001). For example, Carpino et al. (2003) observed vanillin in Ragusano cheeses that they attributed to FA in the milk. This hypothesis has merit given the fact that commercial chemically synthesized vanillin is actually formed from the microbial conversion of FA to vanillin (Muheim and Lerch, 1999;

Mathew and Abraham, 2004). In any event, the impact of phenolic compounds such as FA on the sensory and microbiological attributes of milk and dairy products has not been fully elucidated and is an area worthy of continued research.

The estimated daily intake range of dietary phenolics is 20 mg to 1 g, which is higher than that of vitamin E (Srinivasan, et al., 2007). Due to its potent antioxidant activity, the regular ingestion of FA may provide protection against various diseases caused or exacerbated by oxidative stress including cancer, diabetes, cardiovascular and neurodegenerative diseases (Srinivasan et al., 2007). In a Spanish study investigating the contribution of different beverages to total antioxidant intake of Spaniards, milk was considered the most consumed beverage with a mean daily intake of 318 mL; however, milk only accounted for 4% of daily antioxidant intake (Pulido et al., 2003). A study in Norway that evaluated the total dietary antioxidant intake of 61 adults did not list milk as a major source of antioxidant intake in the adults studied (Svilaas et al., 2004). In both studies, antioxidant intake via coffee accounted for the highest percentage of dietary antioxidants ingested, indicating that there is room for improvement in methods of boosting antioxidant activity of milk.

Antioxidant activity of ferulic acid

Normal physiological processes such as metabolism and the innate immune response produce free radicals such as the superoxide anion (O_2•), hydrogen peroxide (H_2O_2) and the hydroxyl radical (•OH) (Dibner et al., 2011). These metabolites cause damage by attacking lipid membranes, proteins, enzymes and DNA. Thus, antioxidants are needed in balance with pro-oxidants to neutralize these reactive oxygen species. Among the body's endogenous antioxidant defenses are enzymatic antioxidants, protein antioxidants in the intracellular fluids, and low molecular weight chain-breaking antioxidants such as vitamins C, E and A (Dibner et al., 2011). At this

time, we cannot feed enzymatic antioxidants so the main route of counteracting oxidative stress is through the inclusion of dietary antioxidants (Dibner et al., 2011).

Ferulic acid has been identified as an antioxidant of interest because of its ubiquitous form in the plant kingdom, and therefore, its integral part of the human diet via vegetables, fruits and beverages. Ferulic acid is expected to both prevent lipid oxidation in food as well as to prevent free-radical-induced diseases (Kikuzaki et al., 2002). Autoxidation of lipids in milk is an autocatalytic free radical process that can be inhibited by antioxidants that block the formation of free radicals. Linoleic acid is a common feed-derived fatty acid found in milk. Autoxidation of linoleic acid in ethanol-buffer system is one of the simplest conditions of oxidation for the evaluation of antioxidant effects (Kikuzaki et al., 2002). Using this system to evaluate the antioxidant activity of FA, FA esters, *p*-coumaric, caffeic and sinapic acids, Kikuzaki et al. (2002) observed the strongest action associated with FA alkyl esters, followed by FA, which had an antioxidant activity similar to that of α-tocopherol. In other assays of antioxidant activity, caffeic acid exhibited the highest activity (Kikuzaki et al., 2002).

Contribution of ferulic acid to antioxidant activity of milk

Li et al. (2009) compared four infant formulas with freeze-dried human breast milk hydrolysates from six mothers for total phenolic composition and antioxidant activities. They reported three phenolic acids: *p*-hydroxybenzoic acid, *p*-coumaric acid, and FA at levels in infant formulas of 783 to 3,549 µg/kg, 1,449 to 1,652 µg/kg, and 1,447 to 1,561 µg/kg, respectively and levels in human breast milk of 614 to 635 µg/kg, 1,391 to 1,444 µg/kg, and 1,425 to 1,490 µg/kg, respectively (Li et al., 2009). Despite their conclusions that the low levels of these phenolic acids was indication of a relatively small contribution to the total antioxidant activity of milk, Li et al. (2009)

maintained that the consumption of a diet rich in phenolic compounds was still likely to be effective in preventing some disease in newborn infants due to the many health-promoting effects of phenolic compounds. In addition, Friel et al. (2002) postulated that milk antioxidant activity is likely heterogeneous; thus, evaluating any one specific compound will not likely explain milk antioxidant protection. Thus, methods reported in literature usually measure total antioxidant capacity rather than the antioxidant activity generated from one compound.

Antioxidant activity of bovine milk and milk replacers

Comparisons of natural milk to formulated milk replacers for nutritional superiority and associated incidence of disease are not new. Recent literature on the benefits of antioxidants in preventing diseases has generated some research comparing the antioxidant capacity of milk versus formula for human infants. Friel et al. (2002) reported that human breast milk samples (n=17) had increased resistance to oxidative stress and therefore, increased antioxidant protection than specialized premature infant formulas (n=3). However, Li et al. (2009) did not find a conclusive disparity between the antioxidant activities (as measured by DPPH• and ORAC methods) of infant formulas analyzed (n=4) versus human breast milk hydrolysate samples (n=6). These mixed results could be due to the variation in formula composition and formulas tested, breast milk composition due to the diet of the mother, and the different assessments of antioxidant activity.

In terms of dairy calf nutrition, the usefulness of a comparison between bovine milk and a formulated dairy calf milk replacer is quite relevant, given that 69% of the dairy calves in the U.S. are raised on milk replacers (USDA, 2007). Calf milk replacers, however, have not been traditionally formulated for antioxidant activity and there are no known reports of this assessment of calf milk replacers in scientific

literature. The antioxidant activity of bovine milk varies depending upon the standardization and processing of the milk and the assay used to determine total antioxidant activity. The antioxidant activity of an unhomogenized milk sample, (originally 4% fat) that was defatted, purchased at a supermarket in Sweden and analyzed by the ABTS method was 3,777 µmol/L (Chen et al., 2003). Another milk sample analyzed the same way from the same supermarket that was homogenized and had milk fat 0.1% had 4,560 µmol/L antioxidant activity.

The natural antioxidative factors in bovine milk include the antioxidative enzymes, (superoxidative dismutase, catalase and glutathione peroxidase), as well as lactoferrin, vitamins C and E, and carotenoids (Lindmark-Mansson and Akesson, 2000). A variety of factors can influence these native antioxidants. Decreasing the fat content of milk will decrease carotenoid content (Lindmark-Mansson and Akesson, 2000). Seasonality affects the amount of catalase. Insufficient dietary Cu, Zn and B vitamins will limit superoxide dismutase just as insufficient dietary selenium will limit glutathione peroxidase activity (Lindmark-Mansson and Akesson, 2000). Heat treatment such as pasteurization can negatively affect catalase and glutathione peroxidase activity. Water-soluble vitamins like vitamin C are lost during storage and certain heat treatments of milk while fat-soluble vitamins appear to be much more stable (Lindmark-Mansson and Akesson, 2000). Other compounds that are also categorized as low molecular weight compounds are thought to contribute to antioxidant activity (Clausen, 2009). In both human milk and bovine milk, it is recognized that there are unidentified factors in natural milk that seem to work synergistically with the native antioxidants to contribute to overall antioxidant activity (Friel et al., 2002).

Calf milk replacers lack the enzymatic antioxidants - catalase, glutathione peroxidase and superoxide dismutase. The carotenoid content will depend upon the

source and concentration of fats included in the milk replacer. The sources of fat and protein in milk replacer can range from milk to animal to plant sources. Therefore, the majority of antioxidant activity in the milk replacer will likely be derived from the vitamins included as well as the type and amount of fat and protein included. In addition, calf milk replacer antioxidant activity will be susceptible to storage and processing conditions. However, an assessment of antioxidant activity in calf milk replacers has not previously been reported to compare the antioxidant activity of common calf milk replacers with that of natural bovine milk. If a disparity exists, an increase in antioxidant activity of calf milk replacers would be expected to reduce symptoms of diseases, increase recovery rate and further normal development (Friel et al., 2002). This could translate into decreased calf mortality and reduced cost of veterinary care and disease treatment on dairy farms.

Summary

In summary, FA is a compound of interest due to its antioxidant and antimicrobial properties as well as its ubiquitous nature in plants. However, the majority of FA is not in a bioavailable form for absorption when the plant is consumed. When enzymatic combinations are utilized as pretreatments for ruminant forage feeds, an increased amount of FA may be potentially released into a free form in the feed. Increased amounts of free FA in ruminant feeds could have a variety of implications for the animal as well as humans that consume milk produced from a lactating ruminant with increased dietary free FA. The subsequent dissertation chapters address several of the aforementioned research questions including the fate of free FA in ruminants and its effects on rumen microbes, the amount of free FA present in milk of lactating cows administered an oral dosage of FA and the antioxidant activity of bovine milk when compared to calf milk replacers.

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CHAPTER TWO

FERULIC ACID UPTAKE IN RAM LAMBS

ABSTRACT

The objective of this research was to investigate the fate of free ferulic acid (FA) in sheep. Ferulic acid is normally present in plants, bound to the indigestible cell wall. If the FA present in a ruminant diet is released from the cell wall with feed pretreatment methods, FA may be released into the rumen for digestion and/or absorption into the bloodstream. Eight male Dorset x Finn lambs were randomly assigned to one of four treatment concentrations, 0 (control), 3, 6 or 9 g (free FA per d) as part of a replicated 4 x 4 Latin square design. Lambs were housed individually and consumed chopped alfalfa hay (*Medicago sativa)* (22.8% CP, 39.3% NDF, 0.73 Mcal/kg NE_g) *ad libitum* and 350 g corn grain (*Zea mays* L.) (9.1% CP, 11.2% NDF, 1.52 Mcal/kg NE_g) once daily at 0800 h. Basal levels of FA in hay, grain, blood, feces and urine were established following a 14 d adjustment to diet and housing. An oral dose of free FA was administered for 5 d via bolus after each morning feeding, after which hay, grain, blood, feces and urine were sampled. Weekly BW were recorded, and DMI were measured daily. In addition to treatments, each lamb ingested a daily average of 3.78 g FA in its bound form via the offered hay (2.67 mg/g FA; 1.0 kg/d DMI) and corn (3.17 mg/g FA; 0.35 kg/d DMI). Hay DMI was different among treatments (P = 0.04; SE = 56.0 g), but lamb BW was not affected by FA treatment (P = 0.28). Refusals across treatments were not different in NDF level (P = 0.30) or FA concentration (P = 0.82). Fecal FA concentration did not differ among treatments or when compared to basal concentrations (P = 0.53) while urine FA concentration increased as FA dose

increased ($P < 0.01$), indicating that free FA was absorbed and transferred into urine. No free FA was found in the plasma analyzed, suggesting that disappearance from the blood of absorbed free FA occurred within the 5 h that passed between bolus dosage and blood collection. An in vitro analysis was conducted to assess the degree of inhibition of microbial NDF digestion caused by FA supplementation. In vitro NDF disappearance was not inhibited as a result of FA treatment ($P = 0.80$). These data in combination with the results of the lamb study indicate that free FA as 0.24, 0.43 and 0.70 percent of DMI in lambs is absorbed and excreted in the urine as opposed to the feces with no apparent effects on rumen microbial NDF digestion.

Key Words: ferulic acid, in vitro, plasma, sheep, urine, rumen

INTRODUCTION

Bound ferulic acid (FA) is a phenolic acid that inhibits ruminal fiber digestion due to cross-linkages with lignin that link potentially digestible hemicellulose to the largely indigestible lignin complex (Jung and Allen, 1995), rendering it unavailable for microbial digestion. Some microbiota such as *Fibrobacter succinogenes* and *Butrivibrio fibrosolvens* have feruloyl esterases capable of cleaving the ester linkages (Besle et al., 1995) but the process occurs inefficiently in vivo. Activity is greatly enhanced by the addition of xylanases, pectinases, cellulases and other cell wall degrading enzymes (Mathew and Abraham, 2004). Researchers have released FA using multienzymatic combinations in vitro and in incubations from various feedstuffs (Faulds and Williamson, 1995; Kroon and Williamson, 1996; Faulds et al., 2002; Yu et al., 2002). Moreover, often 10 percent and sometimes more than half of fed lignin is solubilized and may be absorbed in ruminants and rabbits (Singleton and Kratzer, 1969), likely due to the absorption of low molecular weight phenolics.

Once FA is in a free form in the rumen, its fate in vivo is largely unknown. Ferulic acid is water-soluble and of low molecular weight (194.2), supporting the idea that it could be carried in the bloodstream to peripheral tissues or in the liquid fraction flowing from the rumen to be excreted in the feces. While extensive ruminal degradation of free FA is unlikely (Besle et al., 1995), and negative effects of phenolics on rumen microbial digestion have been alluded to (Jung, 1988; Jung and Fahey, 1983), FA transport to peripheral tissues could be beneficial due to its antioxidant, anti-inflammatory, and chemopreventative properties (Ou and Kwok, 2004).

Thus, rather than a quantitative collection, the objectives of this study were to

determine if orally dosed FA was absorbed into the bloodstream or if it remained indigestible to be excreted in the feces, and to determine the effects of an oral dose of increments of FA on rumen digestibility and DMI of ram lambs.

MATERIALS AND METHODS

Sheep Feed Trial

***Selection of Subject Animals and Dosage Levels*.** Several studies report the effects of FA administered to rats (Choudhury, 1999; Adam et al, 2002; Zhao, 2004; Ardiansyah, 2008), but there is a lack of studies where oral dosages of FA have been fed to ruminants. Using FA concentration estimates of 2.84 mg FA per g of alfalfa DM (Jung and Fahey, Jr., 1983) and 3.17 mg FA per g of corn as analyzed by Miner Institute (Chazy, NY), combined with the estimated DMI of 1.0 kg/d of alfalfa hay and 0.35 kg/d of corn, the total predicted FA ingested by the ram lambs each day would be 3.95 g/d. Thus, if all of the FA in the lambs' diet was to be released in the rumen, the lambs would have opportunity to metabolize approximately 3.95 g/d of free FA. At the initiation of the study, we slightly underestimated the concentration of FA in our corn, arriving at a total predicted FA ingestion of 3 g/d. Therefore, the dosage concentration of 3 g/d was determined to be representative of the approximate amount of FA present in the basal diet. In a study investigating the bioavailability of FA, rats received diets enriched with FA so that they consumed 10, 50 or 250 μmol/d FA (Adam et al., 2002) which would equate to dosages for a 30 kg ram lamb of 0.40, 1.94 and 9.8 g/d. Since 9.8 g represented the upper limit seen in literature for a non-toxic dosage that would also provide sufficient FA to track its distribution in the body, 9 g was chosen as the upper limit for this study. An intermediate dosage of 6 g was also

given; thus, the dosage concentrations of 3, 6 and 9 g represented approximately 75, 150 and 225 percent of the estimated FA ingested via daily diet.

Experimental Design. Eight newly shorn Dorset x Finn ram lambs from the Cornell Research Farm were allowed 14 d of adaptation to diet and housing prior to the treatment period. During the adaptation and exercise periods, lambs were housed in a group exercise pen in the Cornell Large Animal Research and Teaching Unit (Ithaca, NY). During treatments, they were housed in individual metabolic crates in an environmentally controlled room (23°C). On d 1 of the first treatment period, the mean age of the ram lambs was 102 ± 6.7 d and mean BW was 28.7 ± 1.2 kg. Differences in age or BW were accounted for by the Latin Square experimental design.

A daily ration of 350 g cracked corn, *ad libitum* access to chopped alfalfa hay (39.3% NDF) and a free-choice mineral supplement (Agway, Ithaca) was provided for each lamb in separate feeders (Table 2.1). The diet was formulated to contain 22.5% CP and 34.9% NDF and to meet or exceed the predicted requirements for 30 kg lambs with an expected ADG of 300 g/d (NRC, 2007) using the Cornell Net Carbohydrate and Protein System for Sheep (Cannas et al., 2004). Water was available *ad libitum*.

A replicated, balanced 4 x 4 Latin Square experimental design was implemented so that two lambs were randomly assigned to one of four weekly treatment progressions; treatments consisted of either control, 3, 6, or 9 g of a daily FA bolus for 5 d. Each 5 d experimental period was followed by 2 d of rest, during which lambs were group housed in an exercise pen. During the subsequent treatment period, lambs received the next increment of FA dosage. Lambs were fed daily at 0800 h. Throughout each experimental period, lambs were housed in a metabolic crate designed for total

Table 2.1 Ingredients and the chemical composition of the diet[1] and mineral mix[2]

Item	Alfalfa hay	Cracked corn
DM, %	89.2	88.9
CP, % DM	22.8	9.1
NDF[3], % DM	39.3	11.2
ADF, % DM	30.3	3.8
Lignin, % DM	8.1	1.20
Starch, % DM	1.6	68.7
Crude fat, % DM	2.9	4.4
Ash, % DM	9.17	1.64
NE_M, Mcal/kg	0.59	2.20
NE_G, Mcal/kg	0.33	1.52
Calcium, % DM	1.48	0.01
Phosphorus, % DM	0.33	0.34
Magnesium, % DM	0.30	0.14
Potassium, % DM	2.12	0.43

[1]As analyzed by Dairy One Forage Laboratory Service, Ithaca, NY.
[2]Agway sheep and goat mineral mix with Calcium (18 to 20%), salt (36.5 to 41.5%), Selenium (90 ppm), Zinc (1.6%), Manganese (8,000 ppm), Iron (4,600 ppm), Iodine (70 ppm) and Cobalt (60 ppm).
[3]Analysis at Cornell Animal Science Lab values were 41.1 ± 3.40% for alfalfa and 12.5 ± 1.52% for corn.

collection of urine and feces and each ram lamb received free FA (3, 6, or 9 g) administered via bolus once daily, immediately after feeding; control lambs were not administered FA. Ferulic acid (Sigma-Aldrich, St. Louis, MO; Fisher Scientific Co., Pittsburgh, PA), was encapsulated in Size 000 capsules (Capsuline, Inc, Pompano Beach, FL) and orally dosed with a balling gun (Premier One, Washington, IA). Study protocol and procedures for this experiment were approved by the Cornell University Institutional Animal Care and Use Committee.

Sampling. During the experimental periods, feed refusals were weighed and recorded daily, and feed samples were collected weekly; all were stored at -20°C to be analyzed for FA concentration. Weekly BW were measured to monitor lamb weight gain. Prior to the initial bolus administration on d 1 of each experimental period and later on d 5 approximately 5 h post-dosage, samples were collected of blood, urine, and feces to be analyzed for FA concentration. Approximately 10 mL of blood was collected via jugular puncture using Vacutainer plasma tubes with heparin, (Fisher Scientific, Pittsburgh, PA) and subsequently centrifuged for 20 min at 4°C and 2500 x *g*. Plasma was frozen at -20°C until assayed for FA concentration. Total urine produced in the 5 h period prior to bolus administration on d 1 as well as the 5 h period following bolus administration on d 5 was collected, weighed, and an aliquot was frozen at -20°C until assayed for FA concentration. Total feces excreted by each animal for the 5 h periods before dosage on d 1 and after bolus administration on d 5 were also collected, weighed, and a 10 percent aliquot was frozen until assayed for FA, NDF and ADF concentrations. Five days of FA supplementation preceded the fecal collection.

Analyses. Feed, feed refusals and fecal samples were collected during the experimental periods, weighed, and dried at 60°C for 72 h. All were ground through a

1 mm screen using a Wiley mill and 0.25 g of each was analyzed for NDF using the Ankom Method for Determining Neutral Detergent Fiber (Ankom Technology, Macedon, NY). Sodium sulfite and heat stable alpha amylase were used; heat and agitation cycles were 80 min. Hay and corn were analyzed for composition by Dairy One Forage Laboratory Service (Ithaca, NY).

Feed, feed refusals and fecal samples were also analyzed for FA concentration at Miner Institute (Chazy, NY) according to the following procedures: Ferulic acid was quantified using modified procedures of Iiyama et al. (1990) and Jung and Shalita-Jones (1990). Total (ester- and ether-linked) *p*-coumaric and ferulic acids were extracted from 100 mg of neutral detergent residue with 10 mL of 4 *N* NaOH by microwave digestion (MARS 5 XPRESS microwave and TFM digestion tubes; CEM Corporation, Matthews, NC). Digestion was conducted using a 5 min ramp to 180°C, a 90 s hold at 180°C and a 15 min cool down. The sample was quantitatively transferred to 50 mL high-speed centrifuge tubes with two 5 mL washes of HPLC-grade water. The sample was acidified to a pH of 1.5, refrigerated, centrifuged, filtered, washed, eluted, and stored in a HPLC amber vial at -20°C until it was analyzed.

Identification and quantification of FA in hay, corn, orts, and fecal samples was done on a HPLC system (Varian Prostar; Varian, Inc., Palo Alto, CA) composed of an autosampler (model 410), a dual pump system (model 210), and a diode array detector (model 335). A Spherisorb ODS-2 analytical column (250 mm x 4.6 mm, 5 µm; Waters Corp., Milford, MA) and a Pelliguard LC-18 guard column (2 cm x 4.6 mm; Supelco, Inc., Bellefonte, PA) were used. The solvents were: A) 2% HPLC-grade butanol and 0.3% HPLC-grade glacial acetic acid in HPLC-grade water, and B)

HPLC-grade methanol. The gradient was 100% solvent A for 20 min, 80% solvent A and 20% solvent B for 7 min, and 100% solvent A for 4 min. The flow rate was 1.8 mL/min. Detection was at 320 nm. Integration of HPLC spectra was achieved using Star Chromatography Workstation software (version 6.41; Varian, Inc., Palo Alto, CA). Ferulic acid standard was 4-hydroxy-3-methoxycinnamic acid (99%) from Acros Organics (Morris Plains, NJ). The FA concentration was adjusted for standard purity and losses from the method in Chaves et al. (1982); a recovery factor was used.

An adaptation to the methods of Zhao et al. (2003) was used to analyze plasma and urine samples for FA concentration. Plasma or urine (200 µL) was acidified with 1 *M* acetic acid to reach a pH of 5.0. Then, 40 µL of 2 mmol salicylic acid aqueous solution (internal standard) and 5 µL of distilled water were added. To 50 µL of plasma or urine, 50 µL of 0.1 *M* sodium acetate buffer (pH 5.0) and 900 µL HCl-ethanol (0.05 mol/L) was added. Samples were vortexed 30 s, sonicated 30 s, vortexed 30 s, and subsequently centrifuged for 5 min at 4°C and 5000 x *g*. Supernatant was transferred to a HPLC amber vial (Fisher Scientific Co., Pittsburgh, PA) and frozen at -20°C until HPLC analysis.

Identification and quantification of FA in plasma and urine was done on a HPLC System Gold with 32 Karat software (Beckman-Coulter, Inc., Fullerton, CA) using a C_{18} Spherisorb ODS-2 analytical column (4.6 x 250 mm, 5µm; Waters Corp., Milford, MA) with a Spherisorb ODS-2 guard column (4.6 x 10 mm, 5 µm; Waters Corp., Milford, MA). The solvents were: A) 20% HPLC-grade methanol in 5 mmol/L HPLC-grade hydrochloric acid in HPLC-grade water, and B) HPLC-grade acetonitrile. Solvents were mixed using a linear gradient apparatus by changing solvent B as follows: 0% at 0 min, 15% at 5 min, 25% at 15 min, and 0% at 20 min. The flow rate

was 1.0 mL/min and the detection was at 320 nm. Ferulic acid standard was 4-hydroxy-3-methoxycinnamic acid (99%) from Sigma-Aldrich (St. Louis, MO). Sample identification was confirmed by comparing retention times and absorption spectra to those of standard materials.

In Vitro Study

According to the method of Marten and Barnes (1980) as modified by the Ankom method for in vitro true digestibility using the DAISY^{II} Incubator, Ankom F57 filter bags were pre-rinsed with acetone and 0.25 g of ground (1mm) alfalfa hay from the lamb trial was weighed into each bag. Bags containing alfalfa (n=18) and two blanks were assigned to each of four jars. Rumen fluid (4000 mL) was collected from a forage-fed fistulated dry cow, strained through cheesecloth and purged with carbon dioxide. Lamb rumen volumes were estimated from Russell (2002) and equivalent concentrations of FA were added to 2000 mL of buffer/rumen fluid solution to approximate ruminal conditions of lambs fed 0, 3, 6 or 9 g of FA in vivo. Buffer solutions A and B were prepared separately in advance. Solution A contained the following in quantities of g/L distilled water: 10.0 KH_2PO_4; 0.5 $Mg_2SO_4 \cdot 7H_2O$; 0.5 NaCl; 0.1 $CaCl_2 \cdot 2H_2O$ and 0.5 urea. Solution B contained the following in quantities of g/L distilled water: 15.0 Na_2CO_3 and 1.0 $Na_2S \cdot 9H_2O$. Buffer solutions (50 mL) were mixed (24 mL B to 1 L of A) and either 0, 0.26, 0.52 or 0.78 g of FA was dissolved into 50 mL of buffer solution. Buffer solution (1550 mL), rumen fluid (400 mL) and FA in buffer solution (50 mL) were added to each DAISY jar, representing ruminal conditions of lambs fed 0, 3, 6 or 9 g of FA previously. At each time point of 0, 3, 6, 9, 12, 18, 24, 30 and 48 h, two bags containing alfalfa were removed from each jar, the jar was purged with carbon dioxide and bags were recorded, rinsed with water

followed by neutral detergent solution, wrapped in aluminum foil and frozen at -20°C until NDF analyses. All bags were analyzed for NDF using the Ankom Method for Determining Neutral Detergent Fiber (Ankom Technology, Macedon, NY). Sodium sulfite and heat stable alpha amylase were used; heat and agitation cycles were 80 min. A week later, the in vitro study was replicated.

Statistical Analysis

Data were statistically analyzed as a replicated 4 x 4 Latin square design using the PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC) with treatment, ram, period and square included in the model; ram was treated as a random variable to account for individual variation among animals (Templeman and Douglass, 1999). As an additional comparison, orthogonal contrasts were used to find linear, quadratic or cubic effects due to treatments. Data are reported as least square means ± SEM; effects were considered significantly different when $P < 0.05$.

RESULTS AND DISCUSSION

Physiological Fate of Ferulic Acid

The estimated FA concentration of the alfalfa hay, 2.84 mg FA/g alfalfa hay, was one of the higher alfalfa ferulate estimates in literature, which are typically lower than grass ferulates, but was similar to the analyzed FA concentration, 2.67 mg FA/g alfalfa hay. With an average DMI of 1.0 kg/d of alfalfa hay and 350 g/d corn among the eight ram lambs throughout the course of the study, each lamb ingested a daily average of 3.78 g FA in its bound form via the offered hay (2.67 mg FA/g hay) and corn (3.17 mg FA/g corn). While some of the ester-linked FA could potentially be released in the rumen, it is unlikely that a quantitative amount was released and

metabolized without the assistance of enzymatic treatment or other such feed modifications; moreover, ether-linked FA has not been shown to be successfully hydrolyzed under ruminal conditions. Therefore, a certain concentration of FA was expected in the feces, independent of treatment (Jung and Allen, 1995). The average fecal FA concentration was 1.78 g FA/kg feces. Not only were there no differences (P = 0.53) in fecal FA concentration among treatments or versus the control, but the treatments were also not different from the basal concentrations observed prior to FA administration each treatment week (Table 2.2), suggesting that there was no substantial passage of dosed free FA into the feces. In addition, no free FA was found in the blood analyzed, indicating clearance from the blood of absorbed free FA was rapid and had occurred within the 5 h that passed between bolus dosage and blood collection. Urine FA concentration increased with increasing levels of free FA administered ($P <$ 0.01; Table 2.2). These data indicate that dosed free FA in lambs was absorbed and excreted in the urine; estimated recovery of each FA dosage at 5 hours post-dosage was 0.65% of the 3g dosage, 0.78% of the 6g dosage and 1.12% of the 9g dosage. The less than full recovery of the oral FA ingested by lambs may be accounted for by the incomplete urinary collection, partial ruminal degradation, tissue uptake, and losses of monomeric phenolics during the sample preparation procedure for FA quantification, which consisted of NDF analysis. However, implications of the transfer of even minute amounts of FA or FA degradation products into peripheral tissues could have potential impacts in the animal. For example, trace amounts of FA or FA degradation products that reach the mammary gland of a lactating animal could alter sensory, microbiological and antioxidant properties of the milk (Naim et al., 1988; Ou and Kwok, 2004).

Table 2.2 Concentration of ferulic acid in orts, urine and feces[1,2]

Item	Orts FA concentration, mg/g	Urine FA concentration[3], µm	Fecal FA concentration, mg/g
Basal	---	2.76[a]	1.76
0g (Ctrl)	1.98	1.66[a]	1.89
3g	1.93	100 [ab]	1.75
6g	1.88	240[b]	1.72
9g	2.11	519[c]	1.78
SEM	0.18	41.3	0.11
P value	0.82	< 0.01	0.53
Orthogonal Contrasts			
Linear	0.66	< 0.01	0.37
Quadratic	0.45	0.04	0.25
Cubic	0.74	0.58	0.99

[1]The concentration of ferulic acid in the feed was 2.67 mg/g in the alfalfa hay and 3.17 mg/g in the cracked corn; there was no detectable concentration of ferulic acid in the blood

[2]Within a column, means without a common superscript letter differ, $P < 0.05$

[3]Total amount collected during the 5 h collection period

Similar results were noted in rabbits fed phenolic acids, who excreted the majority of their dose unchanged in the urine (Williams, 1959). During an investigation of the bioavailability of FA in humans from tomato consumption, Bourne and Rice-Evans (1998) observed the peak time for maximal urinary excretion around 7 h post-ingestion and the recovery of FA and feruloyl glucuronide in the urine to be 11–25% of that ingested.

In addition, our observations of the rapid disappearance of orally ingested FA from the blood, the presence of free FA in urine and low recovery of FA in feces concur with those of Zhao et al. (2003). In rats, the orally administered free FA almost completely disappeared from blood plasma by 30 min post administration (Zhao et al., 2003). In our study, the free FA was not detectable by 5 h post-dosage. Zhao et al. (2003) recovered 72 percent of the orally administered FA as free FA or its conjugated forms in the rat urine within 40 h of administration. While we did not perform a total urinary collection, the presence of FA in lamb urine was dose dependent. The difference in our recovery of urinary FA may be explained by losses due to incomplete urinary collections, partial degradation of FA in the rumen and the fact that we accounted for only FA, while the 72 percent recovery Zhao et al. measured included not only free FA, but also its conjugated forms. The amount of FA excreted by the rats in their feces did not differ from the concentration of FA in the feces of the control group (Zhao et al., 2003), as observed with the lambs. It is unlikely that free FA derived from the treatment boluses could have been completely removed from the digestive tract by 5 h. Cherney et al. (1991) observed that lag time for liquid passage (Cobalt dosed) in lambs was no less than 11 h. Furthermore, mean retention time for liquid passage was 37 h. This suggests that if FA passed to feces, a

one-point collection at 5 h after 5 days of pulse dosing free FA, should result in its detection at 5 h.

Effects of Ferulic Acid on Growth and Feed Intake

The projected DMI as a percent of BW is 2.9 for 30 kg ram lambs with targeted BW gain of 300 g/d (National Research Council, 2007). Lambs on the study ate an average of 1 kg/d alfalfa hay plus 350 g corn each day for a total average daily DMI during the course of the 4 wk study of 1.35 kg/d, which was 3.8% of their final average BW. Average BW at the beginning and end of the study was 28.7 and 35.3 kg for an ADG of 210 g/d during the study. The average BW for lambs throughout the four weeks of treatment was not affected by FA supplementation level (P=0.28). All lambs had a net weight gain during the study and there were no significant differences in ADG in response to treatment concentrations, (P=0.10; Table 2.3). Orts (refusals) across treatments were not different in FA concentration (P = 0.82) or NDF level (P = 0.30) (Table 2.2, 2.4). A quadratic effect was observed for DMI (P < 0.01) and orts intake (P = 0.02) with the highest DMI observed when lambs were dosed with 3g and 6g FA; similarly, these lambs had decreased orts (Table 2.4).

Although traditional literature provides examples of feed intake depression due to addition of simple phenolics to diets of rats, and all phenolic acids appear to be at least moderately toxic to animals (Jung et al., 1983), we did not observe a significant depression in feed intake of lambs in the study (Table 2.3). Ferulic acid is one of about 25 phenolics considered to be universal in plant-derived animal diets so appreciable negative physiological activity associated with the amount present in a normal plant diet (3 g) would be surprising (Singleton and Kratzer, 1969). In fact, lambs given twice this amount of free FA (6g) consumed the most feed per day.

Table 2.3 Lamb intake and growth

Treatment	Total avg daily DMI, g/d	Average daily gain, g/d
0g (Control)	1,253	183
3g	1,405	141
6g	1,408	276
9g	1,288	95.0
SEM	59	78.9
P value	0.03	0.10
Orthogonal Contrasts		
Linear	0.55	0.56
Quadratic	< 0.01	0.17
Cubic	0.88	0.04

Table 2.4 Effect of ferulic acid supplementation on NDF content of orts and feces[1]

Treatment	Orts, g/d	Orts NDF, % DM	Fecal NDF, % DM
Basal	---	---	50.6
0g (Control)	199	47.0	50.1
3g	155	48.3	50.8
6g	175	53.6	48.5
9g	223	46.4	48.1
SEM	20	2.8	0.8
P value	0.08	0.30	0.06
Orthogonal Contrasts			
Linear	0.25	0.78	0.02
Quadratic	0.02	0.16	0.44
Cubic	0.64	0.21	0.15

[1]NDF content of the feed on a DM basis was 39.3% of alfalfa hay and 11.2% of cracked corn.

Effects of In Vitro Ferulic Acid Supplementation

Ferulic acid supplementation did not affect NDF disappearance (P = 0.80) of alfalfa at any level of FA concentration used in the in vitro study (Table 2.5).

Essentially every phenolic substance has some antibacterial properties (Singleton and Kratzer, 1969), but the inhibitory activity will vary based on factors such as the bacterial species, the individual compound, the plant species and the pH (Herald and Davidson, 1983; Cherney, 1989). Phenolic-carbohydrate complexes have been shown to inhibit in vitro fermentation of structural carbohydrates (Cherney et al., 1992; Jung, 1988) and suppress rumen bacterial growth (Akin et al., 1988; Griggs et al., 1989).

While phenolic acids and phenolic extracts of maize and barley inhibited digestion of forage, trans-*p*-coumaric acid is consistently identified as the most effective inhibitor, (Herald and Davidson, 1983; Akin et al., 1988). Although FA has been reported as generally antibacterial and antifungal (Barber et al., 2000), there is evidence that rumen microbes can adapt to increased concentrations of FA in vitro (Cherney et al, 1993). Moreover, while bound phenolics may inhibit microbial digestion (Jung and Fahey, Jr., 1983), it is less clear how free phenolic monomers are inhibitory to microbial digestion (Cherney, 1989). Thus, the concentration of FA and length of time that FA would have to be retained in the rumen in order to affect microbial NDF digestion are two questions we sought to answer with the in vitro study. However, the in vitro analysis did not provide evidence of microbial inhibition of alfalfa NDF digestion by any level of FA supplementation, (P = 0.80; Table 2.5). Furthermore, neither the extent (P = 0.14) nor the rate (P = 0.34) of NDF digestion was significantly affected by addition of FA (Table 2.5).

Table 2.5 Rate and extent of 48 h in vitro NDF digestion of ground alfalfa after addition of incremental doses of ferulic acid

Treatment[1]	Extent of digestion[2], %	Rate of digestion, %/h
0g (Control)	56.0[ab]	7.1
3g	53.1[ab]	6.3
6g	60.7[a]	8.5
9g	51.0[b]	6.6
SEM	3.2	0.8
P value	0.14	0.34

[1]In vitro treatments contain ferulic acid added to represent ruminal conditions of lambs fed 0, 3, 6, or 9 g of ferulic acid.
[2]Within a column, means without a common superscript letter differ, $P < 0.05$

Theoretically, monoaromatic compounds like FA are potentially fully degradable in the rumen, but the degradation of FA in the rumen is limited by the low redox potential, the small populations and slow growth of microorganisms that use monoaromatics as well as their limited retention time (Besle et al., 1995). *Ruminococcus albus, Ruminococcus flavefaciens, Bacteroides succinogenes, Butrivibrio fibrisolvens, Streptococcus bovis* and *Wolinella succinogenes* are some of the microbial populations found in the rumen that can perform degradative steps in the breakdown of FA; these cellulolytic bacteria are able to hydrogenate the alkyl chain of FA but are not able to transform it further (Besle et al., 1995). Thus, the FA that did not escape the rumen may have been modified to less toxic forms (Akin, 1988), and the FA derivative most commonly reported in rumen fluid is phenyl-3-propionic acid (Chesson, 1982) in addition to benzoic and cinnamic acids (Cremin, Jr. et al., 1995). The inefficient breakdown of FA could explain why some of the FA transferred to the blood and urine in lambs within 5 h of FA dosage. Given that concentrations of dosed free FA were above 75, 125 and 225 percent of normal dietary levels of primarily unavailable forms of FA, a reduced DMI and NDF digestion may have been expected. Instead, DMI was highest for the 6g dosage. This 6g dosage represented 125 percent of the bound FA ingested in the feed, and NDF forage digestion in vitro was unaffected at all dosage concentrations.

CONCLUSIONS

This study provides evidence that orally dosed free FA in lambs is taken up from the blood by five h post-dosage and excreted in the urine, lending reason to

believe that peripheral tissue uptake of FA in ruminants is possible. There were no negative repercussions on microbial digestion of alfalfa NDF in vitro or lamb DMI as a result of FA administration. Thus, the amount of FA released due to the use of forage pre-treatments would not be expected to have adverse effects on animal intake and growth, but future research where FA is fed over a prolonged period of time would be necessary to make definitive statements regarding the effects of FA supplementation on lamb growth. Future studies should measure the proportion of FA converted by rumen microbes versus the amount taken up by the ruminant.

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CHAPTER THREE

FREE FERULIC ACID UPTAKE IN LACTATING COWS

ABSTRACT

Ferulic acid (FA), a phenolic compound with antioxidant and anti-cancer activities, naturally occurs in plants as one of the building blocks of lignin. Many veins of research have been devoted to releasing FA from the lignin complex in order to improve digestibility of ruminant feeds. Thus, the objective of this research was to investigate the transfer of a given dosage of the free form of FA into the milk of dairy cattle. Six mid to late lactation Holstein cows at the Cornell Teaching and Research Dairy Farm were given 14 d adaptation to diet and stall position. *Ad libitum* access to a total mixed ration based on haylage and maize silage (31.1% NDF containing 5.52 mg/g FA) was provided during the study. A cross over design was implemented so that each cow alternated weekly between Treatment (Trt) and control. On d 1, jugular cannulas and urine catheters were placed in all cows. On d 2, Trt cows received a single dosage of 150 g pure FA powder at 0830 h via their fistula (n=4) or a balling gun for nonfistulated cows (n=2). Plasma, urine, feces, feed, orts, milk and rumen fluid were sampled intensively for the next 36 h and analyzed for FA concentration. On d 8, the cows crossed over and the experiment was repeated. When compared to control, FA administration did not have an effect on DMI, milk yield, milk fat yield, milk protein yield, SCC, or NDF content of orts and feces. The concentration of FA in the feces did not change as a result of FA dosage. As expected, FA concentration increased dramatically upon FA dosage and decreased over time until returning to basal levels in rumen fluid (4 h post-dosage), plasma (5.5 h post-dosage), urine (10 h

post-dosage) and milk (14 h post-dosage). Baseline values for FA in urine and rumen fluid were variable among cows and had an effect on FA concentration in Trt cows. From this study, it is observed that orally ingested FA can be transported into the milk and that the physiological transfer of FA occurs from rumen to milk within 6.5 h or the first milking after dosage. Ferulic acid may impact the functionality of milk due to its antioxidant, anti-cancer, and antibacterial activities. Future research will be required to elucidate whether FA in milk is bioavailable and bioactive, and to evaluate the complete sensory and microbiological impacts of increased FA and FA degradation products in milk.

Key Words: ferulic acid, milk, cow, phenolic

INTRODUCTION

Ferulic acid (FA) is a phytochemical commonly found in fruits, vegetables, beverages and cereals. It is also ubiquitous in forage species as a naturally occurring phenolic compound that functions as one of the building blocks of lignin (Jung and Allen, 1995). Much research has been focused on releasing FA from the lignin complex in order to improve digestibility of ruminant feeds (Faulds et al., 2002; Yu et al., 2002; Mathew and Abraham, 2004) as well as to improve the bioavailability of FA in human diets (Bourne and Rice-Evans, 1998; Adam et al., 2002). The release of increased free FA in the diet could have a variety of implications for dairy cattle especially if the FA was transferred into milk. However, due to the interest in FA as a human dietary supplement, few studies have investigated the absorption of FA in lactating animals or ruminants.

In a preliminary study using ram lambs, oral doses of FA were excreted primarily in the urine, supporting the idea that FA was absorbed into the blood and could therefore be taken up by ruminant peripheral tissues such as the mammary gland, (Soberon et al., 2011a). Because consumer perception of milk as a healthy source of nutrients is vital to the dairy industry, increased FA concentrations in milk could be desirable for multiple reasons. Regular ingestion of FA may provide substantial protection against various oxidative stress related diseases including cancer, diabetes, cardiovascular and neurodegenerative diseases, due to its potent antioxidant activity (Srinivasan et al., 2007). Moreover, the inhibition of oxidation due to natural antioxidants in milk could potentially reduce the autoxidation of milk lipids to prolong the stability and shelf life of milk (Ou and Kwok, 2004).

Because the concentration of FA in milk depends upon its absorption and

tissue distribution, the objective of this study was to track the fate of a given dosage of FA in the dairy cow in order to determine the likelihood of its transfer from ingested feed to milk when available in free form.

MATERIALS AND METHODS

Selection of Subject Animals and Dosage

Study protocol and procedures for this experiment were approved by the Cornell University Institutional Animal Care and Use Committee.

Six multiparous Holstein dairy cows in mid to late lactation with somatic cell counts less than 250,000 cells/mL were selected from the Cornell Teaching and Research Dairy Farm (Harford, NY) herd. Four of the cows were rumen fistulated. On average, cows were 4 years old, 178 DIM and average milk yield and SCC were 48.0 kg/d and 24,000 cells/mL, respectively.

In a previous study, sheep with average BW of 28.7 kg were fed 6g of FA via oral bolus with no negative effects on DMI or ADG (Soberon et al., 2011a); this represented 159% of the ingested FA in the diet. Thus, a dosage of 150 g for cows (600 kg with expected DMI 24 kg) was chosen as a safe dosage of sufficient concentration to detect in the plasma, rumen fluid, milk and urine.

Experimental Design

Prior to the sampling period, cows were given 14 d of adaptation to tie stall location. To allow for individual feed intake measurements, cows were housed in individual tie stalls. *Ad libitum* access to water and a total mixed ration based on haylage and corn silage (31.1% NDF containing 5.52 mg/g FA) was provided during the study and adaptation period (Table 3.1). The diet was not different from the diet

Table 3.1 Chemical composition of the TMR[1,2]

Item	TMR
DM, %	45.9
CP, % DM	15.9
Soluble protein, % of CP	41.9
NDF[3], % DM	32.0
Roughage NDF, % DM	22.2
Digestible NDF, % DM	16.8
Starch, % DM	26.9
Fat, % DM	3.52
NE_L, Mcal/kg	1.65
Sulfur, % DM	0.22
Calcium, % DM	0.84
Phosphorus, % DM	0.43
Magnesium, % DM	0.32
Potassium, % DM	1.04
Copper, mg/kg	17.6
Iodine, mg/kg	1.05
Added Selenium, mg/kg	0.30
Zinc, mg/kg	80.9
Vitamin A, IU/g	6.22
Vitamin E, IU/kg	36.2
Monensin, mg/kg	14.4

[1]Includes 61.4% corn silage, 15.7% hay silage, 12.9% grain concentrate mix, 6.3% corn meal and 3.7% corn distiller's grains
[2]As analyzed by Dairy One Forage Laboratory Service, Ithaca, NY
[3]Analysis at Cornell Animal Science Lab values were 31.1% NDF, 16.6% ADF, 1.86% lignin

used for other cattle in the herd at the same stage of lactation. Cows were fed daily at 0830 h. A cross over design was implemented so that each cow alternated weekly between treatment (Trt) and control. Fistulated (n=4) and non-fistulated (n=2) cows were balanced among treatments. All cows were housed in metabolic tie stalls adjacent to their customary tie stalls during the 3 d sampling period. At the conclusion of the first sampling period on d 3, cows returned to their tie stall and were milked in the farm dairy parlor for the next 4 d before commencing the second wk of treatment.

On d 1 of each experimental period, an indwelling jugular catheter (Tygon Microbore Tubing, Norton Performance Plastic, Akron, OH) and urine catheter (Bard Urologic Catheters, Mohawk Medical, Utica, NY) was placed in each cow. On d 2, Trt cows received a single dosage of 150 g pure FA powder via balling gun and bolus or fistula at 0830 h. Plasma, urine, feces, feed, orts, milk and rumen fluid were sampled intensively for the next 36 h. Ferulic acid (The Lab Depot, Dawsonville, GA) was encapsulated in Size 7 capsules (Torpac, Fairfield, NJ) and orally dosed with a balling gun (Nasco, Fort Atkinson, WI).

Sampling

Prior to the initial FA administration on d 2 of each experimental period, samples were collected of rumen fluid, blood, urine, milk and feces to be analyzed for basal levels of FA concentration. During the sampling periods, feed and feed refusals were weighed and recorded daily; all were stored at -20°C to be analyzed for FA, NDF, ADF and lignin concentration. Feed was also analyzed for composition by Dairy One Forage Laboratory Service (Ithaca, NY) (Table 3.1).

Rumen fluid was collected every 30 min for the first 2 h after FA administration, every hour the next 3 h, and once more at 12 h post-dosage. Rumen

contents were sampled from five standardized locations in the rumen, strained through cheesecloth and the rumen fluid was collected and frozen at -20°C until analyzed for FA concentration.

Blood collection took place every 15 min for the first 2 h after FA administration, every 30 min for the next 4 h, and every h thereafter until 12 h post-dosage. Approximately 5 mL of blood was collected via jugular catheter using a 6 mL syringe preloaded with 100 uL heparinized sterile saline (40 U/mL) and subsequently centrifuged for 20 min at 4°C and 2500 x *g*. Plasma was frozen at -20°C until assayed for FA concentration.

Total urine produced in the 30 h period following FA administration was collected every 2 h for the first 12 h, every 4 h for the subsequent 12 h period and every 8 h until 30 h had passed post-dosage. Urine was collected via 5 gallon polybag (Cornell Dairy Plant, Ithaca, NY), weighed, and an aliquot was frozen at -20°C until assayed for FA concentration.

Milk was collected via bucket milker (DeLaval, Kansas City, MO) three times daily prior to and during the experimental periods. Total milk for each cow was weighed and recorded. Two representative samples were stored in 3 oz polypropylene vials and immediately frozen in liquid N to be subsequently stored at -80°C until assayed for FA concentration. A third sample was collected and analyzed for milk composition at Dairy One Laboratory (Ithaca, NY).

A subsample of feces was collected every 6 h for 36 h, weighed, and frozen until assayed for FA, NDF, ADF and lignin concentrations.

Analyses

Feed, feed refusals, rumen fluid, blood, urine, milk and feces were analyzed for

basal and treatment levels of FA concentration using HPLC methods that best suited each sample matrix. Feed, rumen fluid, feed refusals and fecal samples were collected during the experimental periods, weighed, and dried at 60°C for 72 h. All were ground through a 1 mm screen using a Wiley mill and 0.25 g of feed, feed refusals and fecal samples were analyzed for NDF using the Ankom Method for Determining Neutral Detergent Fiber (Ankom Technology, Macedon, NY). Sodium sulfite and heat stable alpha amylase were used; heat and agitation cycles were 80 min.

Feed, feed refusals, rumen fluid and fecal samples were also analyzed for FA concentration at Miner Institute (Chazy, NY) according to the following procedures as described by Soberon et al. (2011a). Ferulic acid was quantified using modified procedures of Iiyama et al. (1990) and Jung and Shalita-Jones (1990). Total (ester- and ether-linked) FA was extracted from 100 mg of ND residue with 10 mL of 4 *N* NaOH by microwave digestion (MARS 5 XPRESS microwave and TFM digestion tubes; CEM Corporation, Matthews, NC). Digestion was conducted using a 5 min ramp to 180°C, a 90 s hold at 180°C and a 15 min cool down. The sample was quantitatively transferred to 50 mL high-speed centrifuge tubes with two 5 mL washes of HPLC-grade water. The sample was acidified to a pH of 1.5, refrigerated, centrifuged, filtered, washed, eluted, and stored in a HPLC amber vial at -20°C until it was analyzed.

Identification and quantification of FA in TMR, orts, rumen fluid and fecal samples was done on a HPLC system (Varian Prostar; Varian, Inc., Palo Alto, CA) composed of an autosampler (model 410), a dual pump system (model 210), and a diode array detector (model 335). A Spherisorb ODS-2 analytical column (250 mm x 4.6 mm, 5 µm; Waters Corp., Milford, MA) and a LC-18 guard column (2 cm x 4.6

mm; Supelco, Inc., Bellefonte, PA) were used. The solvents were: A) 2% HPLC-grade butanol and 0.3% HPLC-grade glacial acetic acid in HPLC-grade water, and B) HPLC-grade methanol. The gradient was 100% solvent A for 20 min, 80% solvent A and 20% solvent B for 7 min, and 100% solvent A for 4 min. The flow rate was 1.8 mL/min. Detection was at 320 nm. Integration of HPLC spectra was achieved using Star Chromatography Workstation software (version 6.41; Varian, Inc., Palo Alto, CA). Ferulic acid standard was 4-hydroxy-3-methoxycinnamic acid (99%) from Acros Organics (Morris Plains, NJ). The FA concentration was adjusted for standard purity and losses from the method in Chaves et al. (1982) and a recovery factor was used.

An adaptation to the methods of Zhao et al. (2003) was used to analyze plasma and urine samples for FA concentration as described by Soberon et al. (2011a). Plasma or urine (200 µL) was acidified with 1 *M* acetic acid to reach a pH of 5.0. Then, 40 µL of 2 mmol salicylic acid aqueous solution (internal standard) and 5 µL of distilled water were added. To 50 µL of plasma or urine, 50 µL of 0.1 *M* sodium acetate buffer (pH 5.0) and 900 µL HCl-ethanol (0.05 mol/L) were added. Samples were vortexed 30 s, sonicated 30 s, re-vortexed 30 s, and subsequently centrifuged for 5 min at 4°C and 5000 x *g*. Supernatant was transferred to a HPLC amber vial (Fisher Scientific Co., Pittsburgh, PA) and frozen at -20°C until HPLC analysis.

Identification and quantification of FA in plasma and urine was done on a HPLC System Gold with 32 Karat software (Beckman-Coulter, Inc., Fullerton, CA) using a C_{18} Spherisorb ODS-2 analytical column (4.6 x 250 mm, 5µm; Waters Corp., Milford, MA) with a Spherisorb ODS-2 guard column (4.6 x 10 mm, 5 µm; Waters Corp., Milford, MA). The solvents were: A) 20% HPLC-grade methanol in 5 mmol/L

HPLC-grade hydrochloric acid in HPLC-grade water, and B) HPLC-grade acetonitrile. Solvents were mixed using a linear gradient apparatus by changing solvent B as follows: 0% at 0 min, 15% at 5 min, 25% at 15 min, and 0% at 20 min. The flow rate was 1.0 mL/min and the detection was at 320 nm. Ferulic acid standard was 4-hydroxy-3-methoxycinnamic acid (99%) from Sigma-Aldrich (St. Louis, MO). Sample identification was confirmed by comparing retention times and absorption spectra to those of standard materials.

Milk sample preparation methods for FA analysis were implemented according to R.H. Liu (Cornell University, Ithaca, NY, personal communication). To prepare milk for the analysis of FA concentration, raw, whole milk samples were thawed in a water bath without light, and centrifuged 15 min at 4°C and 2500 x *g*. The upper fat layer was removed and 4 mL were pipetted into a 50 mL centrifuge tube (Fisher Scientific, St. Louis, MO). Two milliliters HPLC-grade hexanes (Fisher Scientific, St. Louis, MO) were added to the milk, and the sample was vortexed 5 min, centrifuged 5 min at 4°C and 2500 x *g* and the hexane layer was removed to extract lipids; this was repeated. Samples were digested for one h with 11 mL of HPLC-grade 2M sodium hydroxide (Fisher Scientific, St. Louis, MO) on a vortex. Samples were acidified to pH 2.0 with 1.875 mL of 12M hydrochloric acid (Fisher Scientific, St. Louis, MO). Ethyl acetate extraction was performed five times where 6 mL HPLC-grade ethyl acetate was added to the acidified, defatted milk; sample was vortexed 5 min, centrifuged 5 min at 4°C and 2500 x *g*, and the ethyl acetate layer was collected and pooled. The ethyl acetate fraction was evaporated to dryness using a rotary vacuum evaporator in a water bath at 46°C. Sample was reconstituted in 70% methanol/water and transferred to an amber HPLC vial (Fisher Scientific, St. Louis, MO), where it

was stored at -40°C until analysis.

Identification and quantification of FA in milk was done on a tandem HPLC (Ultimate 3000; Dionex, Corp., Sunnyvale, CA) MS (4000 Q Trap with Turbo Ionspray source; AB/Sciex, Foster City, CA) system using a Vydac C18 column, (1 x 150mm, 5um, 300A; The Separations Group, Inc., Hesperia, CA). A binary gradient system consisting of solvent A: 0.1% Optima MS grade formic acid (Fisher Scientific, Pittsburgh, PA) in Milli-Q water (Millipore Corp., Bedford, MA) and solvent B: 0.1% Optima MS grade formic acid in 95% Optima MS grade acetonitrile/Milli-Q water was used in linear gradient mode: 5-5-40-90-90-5-5 percent B for 0-2-22-23-28-29-49 min. The flow rate was 75 µL/min and the column temperature was maintained at 30°C. The mass spectrometer was operated in negative ion MRM-IDA mode with settings: IS = -4200V, CUR = 30, GS1= 20, GS2 = 35, Interface Temp = 280°C, CAD = Medium. MRM transition ions for quantification were optimized using standard compounds: 4-hydroxy-3-methoxycinnamic acid, (ferulic acid; Acros Organics, Morris Plains, NJ) and alpha-cyano-4-hydroxycinnamic acid, (CHCA; Aldrich Chemical, Corp., Milwaukee, WI) for use as the internal standard. Peaks for quantification were determined by matching retention times with MRM transition ion pairs, Q1 mass = 193/Q3 mass = 134 for FA and Q1 mass = 188/Q3 mass = 93 for CHCA, and identity was confirmed by comparing CID (MS/MS) spectra with those obtained from the standard compounds. UV absorbance data was also acquired at 214 and 280 nm but was not useful for quantification or identification purposes. Ten milliliters were injected for quantification and dilutions made as necessary so the analyte signal fell in the linear range of the calibration curve.

Statistical Analysis

Data were statistically analyzed using the PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC) with treatment, cow and period included in the model; cow was treated as a random variable to account for individual variation among animals and each cow served as her own control to further minimize variation. In the analysis of rumen fluid and urine, significant interactions were included for the effects of basal FA concentrations on treatment concentrations. Data are reported as least square means ± SEM; effects were considered significantly different when $P < 0.05$.

RESULTS AND DISCUSSION

Ferulic Acid Distribution in Feed and Feces

The 150 g dosage of FA represented 121 percent of the average FA ingested via TMR (g FA per cow); cows ate an average of 22.8 kg DM and the TMR contained 5.52 mg/g FA (Table 3.2). Using multienzymatic cocktails, researchers have reported between 65 and 95 percent release of FA from various feedstuffs after incubation (Faulds and Williamson, 1995; Faulds et al., 2002; Yu et al., 2002), so the 150 g dosage exceeded what might be released with the use of feed pretreatments. However, it was sufficient to track the distribution of FA throughout the body.

In agreement with results of a preliminary study where FA was dosed to ram lambs (Soberon et al., 2011a), there was no effect of FA administration on fecal or orts FA concentration (P=0.40; P=0.06), DMI (P=0.59), NDF, ADF or lignin content of orts (P=0.46; P=0.46; P=0.49) or NDF, ADF or lignin content of feces (P=0.39; P=0.85; P=0.72) (Table 3.2). Although lignin is often described as indigestible, the use of lignin as a marker for digestion is viewed with some caution due to incomplete

Table 3.2 Distribution of bound ferulic acid

Item	Treatment[1]	Control	SEM	*P* value
TMR[2], g	124.2	126.4		0.59
Orts				
NDF, %	29.8	28.9	1.1	0.46
ADF, %	16.7	16.0	0.8	0.46
Lignin, %	1.81	1.66	0.19	0.49
Ferulic acid, mg/g	6.06	5.62	0.15	0.06
Feces				
NDF, %	53.6	53.0	0.7	0.39
ADF, %	29.7	29.6	0.6	0.85
Lignin, %	6.06	6.14	0.22	0.72
Ferulic acid, mg/g	5.91	5.63	0.33	0.40

[1]Treatment cows were administered a 150 g dosage of ferulic acid

[2]The TMR contained 31.1% NDF, 16.6% ADF, 1.86% lignin and 5.52 mg/g ferulic acid; average DMI was 22.5 kg for Treatment and 22.9 kg for Control

lignin recoveries. These incomplete recoveries have been explained in part by apparent digestion due to the formation of soluble lignin-carbohydrate complexes that pass from the rumen and are not recovered in fecal residues, the partial destruction of the fecal lignin fraction by reagents used in analyses, as well as physical and/or chemical differences between the substances defined as lignin in feed versus feces (Muntifering, 1982).

In a study investigating the apparent digestibility of lignin in the digestive tract of sheep, Fahey, Jr. et al. (1980) attributed this effect to the disappearance of phenolic monomers such as *p*-coumaric acid, FA, and vanillin. They compared the concentration of FA in the lignin fraction of feed and feces for various roughages, and expressed support for the idea that the formation of soluble lignin-carbohydrate complexes by rumen microbial action on grasses could account for the apparent digestion of approximately 50 percent of the total lignin intake. When comparing the µg FA per g of lignin in the TMR versus feces of cows in our study, we observed 66 percent disappearance of µg FA per g of feed in the feces. This would suggest that rumen microbial esterases were efficient in releasing some of the feed-derived FA as described in Fahey, Jr. et al. (1980). This also may explain some of the high concentrations of FA in the cows' urine.

Ferulic Acid Distribution in Rumen Fluid

Ruminal fluid FA concentration increased dramatically within 30 min of the dosage and declined steadily thereafter, returning to basal levels between 3 and 4 h post-dosage (Figure 3.1). Therefore, by 4 h post-dosage, the 150 g FA dosage had yielded to one of the following fates: absorption through the rumen wall into the blood, degradation in the rumen or it had washed out of the rumen with liquid flow to

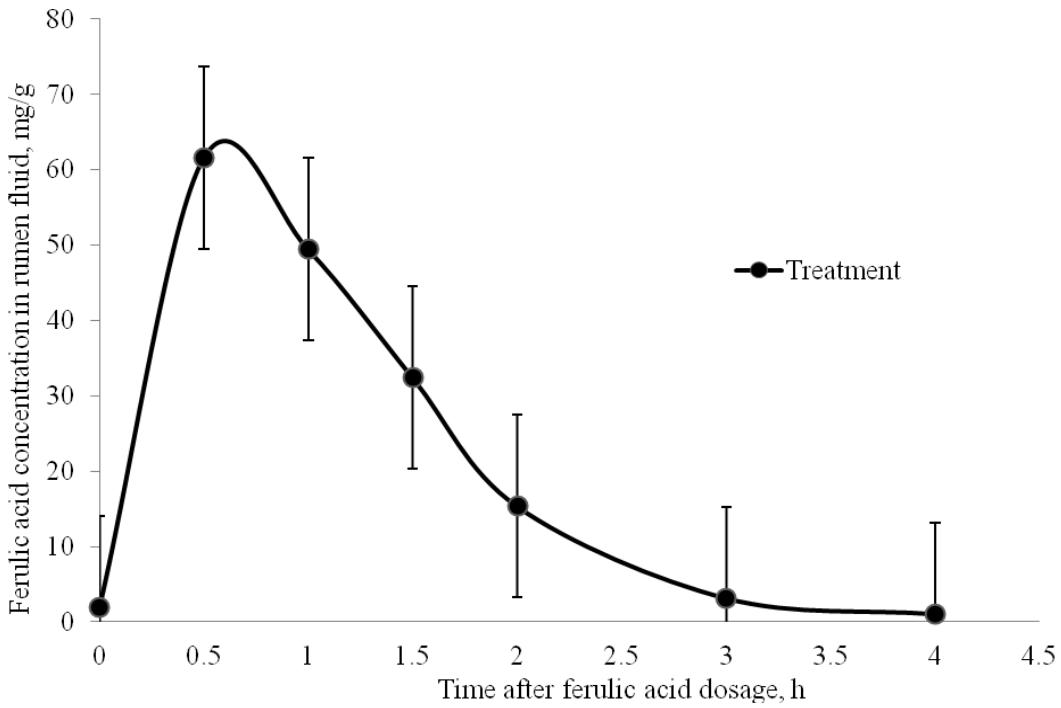

Figure 3.1 Dried ruminal fluid ferulic acid concentration over time for fistulated cows; during treatment, cows (n=4) received a dosage of 150 g ferulic acid immediately following the 0 minute rumen fluid sample.

Certain cellulolytic bacteria are able to perform the preliminary degradative steps in the breakdown of FA but the complete breakdown would require a ruminal retention time that is not physiologically feasible as well as larger populations of these bacteria (Besle et al., 1995). The most likely degradation products of FA are phenyl-3-propionic, benzoic and cinnamic acids (Chesson, 1982; Cremin Jr. et al., 1995) and of these, the most likely to appear in the blood are benzoic and phenylpropionic acids (Cremin Jr. et al., 1995).

Ferulic Acid Distribution in Plasma and Urine

Absorption of FA from the rumen began almost immediately, with the peak plasma FA concentration at the first sample post-dosage, 15 min after the FA administration (Figure 3.2). The concentration of FA in plasma of the treatment cows returned to basal concentrations by 5.5 h post-dosage (Figure 3.2). Similarly, within 5 h of FA dosage in ram lambs orally administered 3, 6 or 9g of FA, FA was no longer present in the blood (Soberon et al., 2011a) and for rats, orally administered free FA had almost completely disappeared from blood plasma by 30 min post-dosage (Zhao et al., 2003).

Following its absorption in the blood, FA was excreted in the urine at levels significantly higher than basal levels by 45 min post-dosage, peaking at 2.75 h post-dosage (Figure 3.3). This compares with a peak time of maximal urinary excretion in humans fed FA from tomatoes at 7 h post-ingestion (Bourne and Rice-Evans, 1998). The difference may be explained by the use of urinary catheters in the cows, which allowed the collection of urine at nearly the same time it was produced whereas in the human study, individuals were allowed to urinate at their discretion, allowing for variation in the time of collection. By 14 h post-dosage, both urine and milk FA

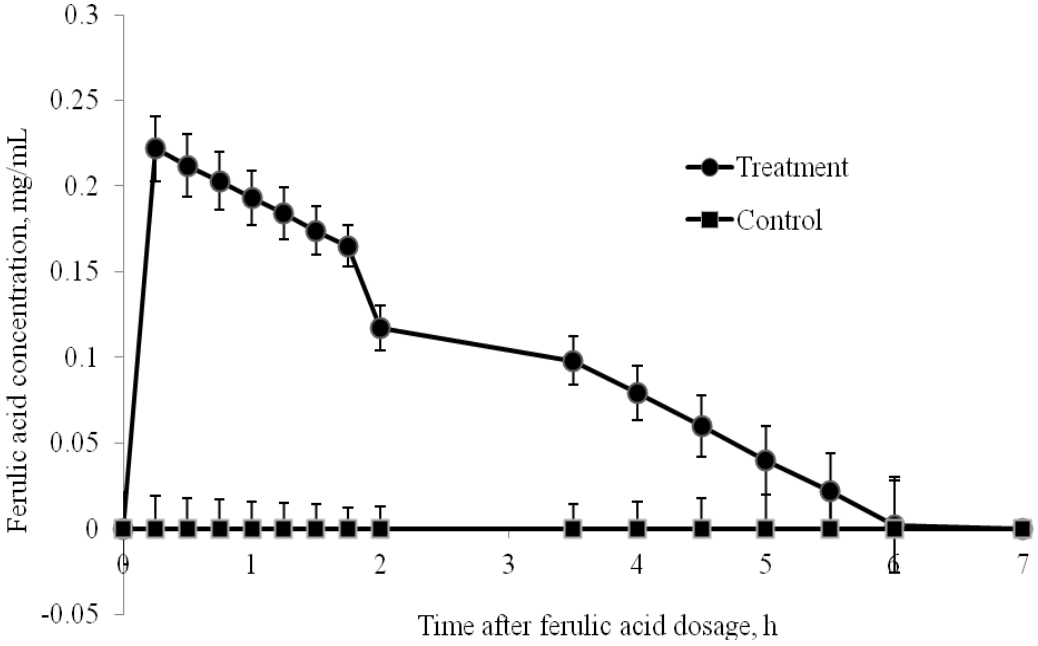

Figure 3.2 Plasma ferulic acid concentration over time for control vs treatment; during treatment, cows (n=6) received a dosage of 150 g ferulic acid immediately following the 0 minute blood sample.

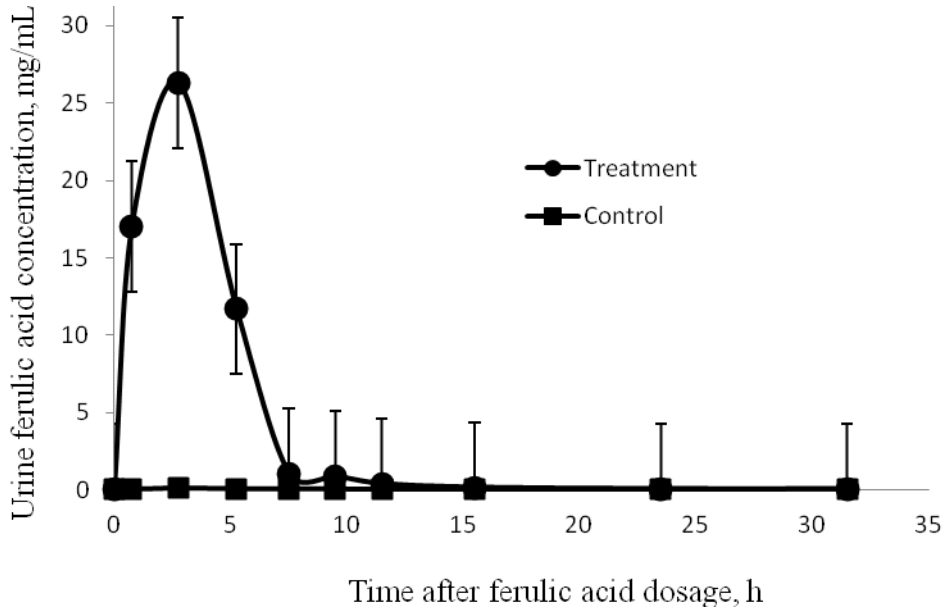

Figure 3.3 Urine ferulic acid concentration over time for control vs treatment; during treatment, cows (n=6) received a dosage of 150 g ferulic acid immediately following the 0 hour urine sample.

concentrations had returned to basal levels (Figure 3.3, 3.4).

In a study where humans were fed a polyphenol-rich diet, and plasma and urine were sampled frequently, great individual variation in absorption and metabolism of dietary polyphenols was observed (Rechner et al., 2002). Similarly, although the same general trend of increased FA in bovine plasma and urine was observed post-dosage, followed by a gradual decline in FA concentration, individual variation in each cow was also observed. Part of the variation could be explained in the statistical analysis by the effect of individual basal FA levels prior to FA administration.

Ferulic Acid Distribution in Milk

There was no effect of FA administration on milk yield (P=0.50), milk fat yield (P=0.46), milk protein yield (P=0.96) or SCC (P=0.50) (Table 3.3). Similar to urine FA concentration, basal levels were achieved by 14 h post-dosage (Figure 3.4).

While we can know with confidence that the peak excretion of FA in milk occurred within 6 h of the FA dosage, it is difficult to accurately gauge the exact time of peak FA excretion because milk was pooled in the cistern continuously until its collection, which was coordinated with the 3x milking schedule.

The average basal concentration of FA in milk was 4.1 µg/L. Besle et al. (2010) observed concentrations of FA in milk of 3.9 µg/L in cows fed ryegrass hay, 0.9 µg/L in cows fed grassland hay and 14.7 µg/L in cows fed grassland pasture. The authors stated that this was the first observation to their knowledge where FA was identified in milk (Besle et al., 2010). In cows fed maize silage, such as in the present study, there is a certain amount of free FA expected from the ensiling process in addition to that which was released by microbial esterases in the rumen (Ostrander et

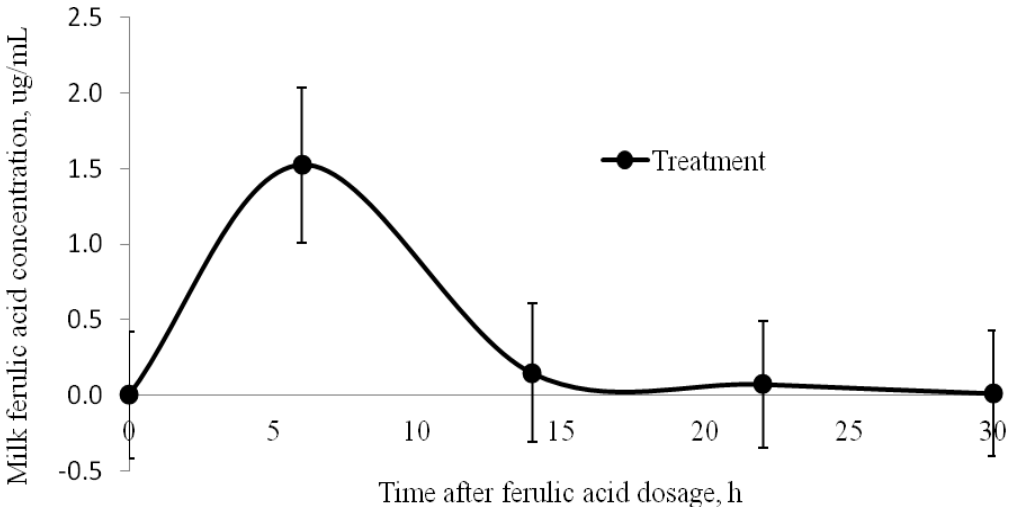

Figure 3.4 Average ferulic acid concentration (µg/mL) in milk at each milking before (0 h) and after dosage of 150 g ferulic acid.

Table 3.3 Effect of ferulic acid dosage on milk composition and yield[1]

Item	Treatment[2]	Control	SEM	*P* value
Fat, %	3.70	3.71	0.22	0.96
Fat Yield, g	1,263	1,323	79	0.46
True Protein, %	3.00	2.87	0.06	0.05
True Protein Yield, g	1,062	1,065	53	0.96
SCCx1000, cells/mL	79.4	64.6	21.2	0.50
Yield, kg	36.6	37.8	1.9	0.50

[1]All values represent daily averages of 3 milkings per day

[2] Treatment cows were administered a 150 g dosage of ferulic acid

al., 1999).

The origin of FA in the milk of control cows can be attributed to this ruminal degradation of soluble polyphenols and cell wall aromatics, which are then absorbed through the rumen and intestinal mucosa, and are subsequently excreted in urine or milk (Scheline, 1991).

While some of the ingested FA escaped the rumen to be transferred into milk, as described previously, it is likely that some of the FA in the rumen was transformed by rumen bacteria into phenyl-3-propionic, phenylacetic, cinnamic or benzoic acids (Chesson, 1982; Cremin Jr. et al., 1995). These precursors can result in increased hippuric acid concentrations in milk, as described in Besle et al. (2010), who observed elevated hippuric acid in milk of dairy cows as a result of consuming diets rich in polyphenols. The potential impact of increased hippuric acid in milk designated for cheese production is important to consider, given that hippuric acid has been shown to be a precursor in the synthesis of benzoic acid by lactic acid bacteria in milk (Sieber et al., 1995). Benzoic acid can impact cheese flavor and act as a natural preservative in cheeses.

Impact of Ferulic Acid in Milk

Ferulic acid may impact the functionality of milk in terms of antioxidant, cholesterol-lowering, antimicrobial, anti-inflammatory, anti-thrombosis and anti-cancer activities in addition to acting as a potential chemopreventive agent against coronary heart disease (Ou and Kwok, 2004) and Alzheimer's disease (Yan et al., 2001). An average of 7.28 mg of FA was recovered in the milk within 30 h of dosage for treatment cows compared to 0.10 mg for control cows. The peak concentration of FA in milk post-dosage was 1.5 µg/mL and the basal concentration prior to FA dosage

was 0.004 µg/mL (Figure 3.4).

Increased antioxidant activity is the primary health benefit expected from milk with increased FA concentrations. The antioxidant activity of FA is comprised of its high scavenging activity for hydrogen peroxide, superoxide, hydroxyl radical and nitrogen dioxide free radicals as well as its inhibition of enzymes that produce free radicals, and ability to increase the activity of enzymes that scavenge free radicals (Ou and Kwok, 2004). The antioxidant activity of natural bovine milk from cows fed the same maize silage-based TMR as the cows in this study, was measured at 52.7 µmol of vitamin C equivalents per mL of milk (Soberon et al., 2011b). In a study that investigated the contribution of different beverages to total antioxidant intake in the Spanish diet where the most consumed beverage was milk (mean daily intake of 317.8 mL), coffee accounted for the highest percentage of daily antioxidant intake at 66 percent, followed by red wine (16 percent) with milk accounting for 4 percent of daily antioxidant intake (Pulido et al., 2003). A similar study in Norway comparing total dietary antioxidant intake of 61 adults also found coffee (mean daily intake of 480 mL) was the major source of antioxidant intake (66 percent) followed by fruits, tea, cereals, wine and vegetables, which contributed 26, 25, 13, 10 and 6 percent of total dietary antioxidants, respectively (Svilaas et al., 2004). These studies indicate that there is room for improvement of the antioxidant activity of milk.

At 200 µM in an ethanol-buffer solution of linoleic acid, FA was the most effective hydroxycinnamic acid tested, with an antioxidant activity similar to that of α-tocopherol (Kikuzaki et al., 2002). In addition, cows in our study received a 0.25 g/kg dosage of FA; rats fed a FA dosage of 0.1 g/kg had significantly increased activities of detoxifying enzymes like glutathione *S*-transferase and quinine reductase (Kawabata et

al., 2000).

Due to its antimicrobial activity towards a wide spectrum of Gram-positive and Gram-negative bacteria (Ou and Kwok, 2004), FA also has potential microbiological effects in milk. Not only do O'Connell and Fox (2001) detail the usefulness of phenolic compounds for increasing shelf life and stability of milk, but they report that FA specifically inhibited the growth of pathogenic bacteria with little ramifications on lactic acid bacteria in milk.

Increased FA and its degradation products may also have a potential impact on sensory properties of milk and cheese. Even at the peak concentration of FA in milk observed in this study (1500 µg/L), which is 121 percent of what was present in the diet, FA is far below the detection threshold measured by Work and Camire (1996) of 62,000 µg FA/L of deionized water. However, an indirect flavor impact of increased FA could result from the direct decarboxylation of FA into 4-vinylguaiacol, which has been observed in the gut of monogastrics and under anaerobic conditions (Besle et al., 1995). 4-vinylguaiacol produced from degraded free FA during storage is responsible for detrimental off-flavors in citrus juices (Naim et al., 1988; Fallico et al., 1996). In beer, the same FA degradation product is considered an essential flavor contributor inherent to the beer production process in beers made with wheat or wheat malt (Coghe et al., 2004; Vanbeneden et al., 2007). Further research is required to investigate the presence of 4-vinylguaiacol in milk with increased FA concentration as well as its potential sensory impact in fermented milk products.

CONCLUSIONS

When compared to control, FA administration did not have an effect on DMI,

milk yield, milk fat yield, milk protein yield, SCC, NDF content of orts or NDF content of feces. The concentration of FA in the feces did not change as a result of FA dosage. As expected, FA concentration increased dramatically upon FA dosage and decreased over time until returning to basal levels in rumen fluid (4 h post-dosage), plasma (5.5 h post-dosage), urine (10 h post-dosage) and milk (14 h post-dosage). Baseline values for FA in urine and rumen fluid were variable among cows and had an effect on FA concentration in treatment cows. From this study, it is observed that orally ingested FA can be transferred into milk and that the physiological transfer of FA occurs from rumen to milk within 6.5 h or the first milking after dosage. Future research will be required to elucidate whether the increased FA concentration in milk is bioavailable and bioactive, and to evaluate the complete sensory and microbiological impacts of increased FA and FA degradation products in milk.

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CHAPTER FOUR

ANTIOXIDANT ACTIVITY OF CALF MILK REPLACERS

ABSTRACT

A calf milk replacer (CMR) is designed to replace whole saleable milk as a lower cost nutrient source for calves while striving to nourish a newborn calf, reduce calf mortality, strengthen immunity and increase animal life span and productivity. Antioxidants (AO) can enhance immune defense by reducing oxidative damage, but CMR are traditionally not formulated for AO activity. The objective of this study was to compare total AO activities of bovine milk with six CMR, varying in amount and source of fat and protein. CMR was donated by Milk Products, LLC. Milk was obtained from the Cornell Dairy Research Farm bulk tank, representing milk produced within 24 h by 455 cows. Milk replacers were mixed to 150 g/L with 40°C, purified water. All samples were extracted in triplicate. Following hexane lipid extraction, both milk and CMR samples were extracted 5 times with ethyl acetate, and then evaporated and reconstituted with 70% methanol/water. Samples were assessed for total AO activity using the peroxyl radical scavenging capacity assay where each sample was diluted to 5 descending concentrations, plated in triplicate. Ascorbic and gallic acids were standards for each plate. Type of protein (soy) had a positive effect on AO activity for CMR A, which exhibited the highest total AO activity. Natural bovine milk had the second highest AO activity. There are many factors that may explain the difference in AO activity between natural milk and formulated CMR, including fat, vitamin and mineral content, enzymatic AO, phenolics and flavonoids, fatty acid profile and AA composition. When comparing AO activity of CMR, it is important to

consider the diversity in feeding recommendations, which will alter the amount of vitamin and mineral content, thus influencing AO activity. Opportunity exists to enhance AO activity of CMR to more closely mimic the AO activity of bovine milk. Future research is warranted to compare a broader range of CMR using methods that account for total lipophilic and hydrophilic AO activities, as well as to investigate the effect of additional compounds in milk that may impact AO activity.

Key Words: antioxidant activity, milk, calf, milk replacer

INTRODUCTION

It has been shown that early life nutrition of a calf can potentially impact not only performance and survival during the time of liquid feeding, but future milk production once the calf reaches adulthood (Soberon et al., 2011a). A calf milk replacer (CMR) is designed to mimic the nutritional benefits of bovine milk in an effort to nourish a newborn calf, reduce calf mortality, strengthen immunity, and increase animal life span and productivity. Antioxidants (AO) can enhance immune defense by reducing oxidative damage, which may translate into decreased calf mortality and increased performance. Research has shown that in humans, breastmilk is significantly higher in AO activity than infant formulas (Friel et al., 2002). However, CMR are not traditionally formulated to take in consideration the dietary AO consumed by the calf.

Scientific literature regarding AO in bovine milk targets the impact of lipid peroxidation on milk quality for human consumption rather than potential effects on calf performance. In lactating cows, feeding dietary AO has been found to reduce mastitis (Smith et al., 1984) and other common transition disorders that may be caused by or exacerbated by oxidative stress (Brzezinska-Slebodzinska et al., 1994; Bradford, 2009). However, research has verified the benefits of dietary AO at times in life when oxidative damage is most severe, such as the first 2 wk postpartum (Friel et al., 2002).

The immature immune system of a calf is often exposed to a variety of disease challenges in the first few weeks of life and the the first immune response of an animal during a disease challenge is to generate reactive oxygen metabolites in an effort to kill the bacteria (Thomas and Kalyanaraman, 1997). Given that oxidative stress has been associated with gut barrier failure (Dibner et al., 2011), there are obvious

negative implications on calf health to having an oxidative imbalance. However, dietary AO can be used to reduce the load of peroxide radicals in the diet by reacting with the free radicals to convert them to less reactive compounds (Thomas and Kalyanaraman, 1997). This strategy was shown to be effective in reducing the oxidative damage of neonatal ileum tissue in birds (Dibner et al., 1996).

With 69% of dairy calves in the United States raised on artificial CMR preweaning (USDA, 2007), the opportunity to better fortify postnatal nutrition in a calf could greatly impact the dairy industry. Therefore, the objective of this study was to determine the total AO activities of bovine milk and six CMR, varying in amount and source of fat and protein.

MATERIALS AND METHODS

Samples

Milk (29% fat, 29% CP on DM basis) was obtained from the Cornell Dairy Research Farm (Harford, NY) bulk tank, representing milk produced within 24 h by 455 cows and frozen in 3 oz polypropylene vials at -80°C. Calf milk replacers (CMR) representing various fat and protein contents and sources were donated by Milk Products, LLC (Chilton, WI). Fat contents of CMR ranged from 15 to 20% DM and were derived from either animal or vegetable fats (Table 4.1). Protein ranged from 20 to 28.5% DM and was derived from milk, soy or animal proteins (Table 4.1). CMR were formulated and shipped directly after mixing to ensure that all CMR were fresh, with similar shelf life.

Sample Preparation

Milk and CMR sample preparation methods were implemented according to

R.H. Liu (Cornell University, Ithaca, NY, personal communication) as described previously by Soberon et al. (2011b). Raw, whole milk samples were thawed in a water bath without light, and centrifuged 15 min at 4°C and 2500 x *g*. The upper fat layer was removed and 4 mL were pipetted into a 50 mL centrifuge tube (Fisher Scientific, St. Louis, MO). Mixing to 15 percent solids is standard for CMR in the dairy industry. Thus, all CMR were reconstituted to 150 g/L with 40°C, purified water. Two milliliters HPLC-grade hexanes (Fisher Scientific, St. Louis, MO) were added to the milk or CMR sample, and the sample was vortexed 5 min, centrifuged 5 min at 4°C and 2500 x *g* and the hexane layer was removed to extract lipids; this was repeated. Samples were acidified to pH 2.0 with 0.5 mL of 12 M hydrochloric acid (Fisher Scientific, St. Louis, MO). Ethyl acetate extraction was performed five times where 6 mL HPLC-grade ethyl acetate was added to the acidified, defatted milk; sample was vortexed 5 min, centrifuged 5 min at 4°C and 2500 x *g*, and the ethyl acetate layer was collected and pooled. All samples were extracted in triplicate. The ethyl acetate fraction was evaporated to dryness using a rotary vacuum evaporator in a water bath at 46°C. Sample was reconstituted in 70% methanol/water and transferred to an amber HPLC vial (Fisher Scientific, St. Louis, MO), where it was stored at -40°C until analysis.

PSC Assay

The AO activity of milk and CMR samples was determined using a peroxyl radical scavenging capacity assay (PSC) described previously (Adom and Liu, 2005; He and Liu, 2006). Each sample was diluted to five descending concentrations, and plated in triplicate; ascorbic and gallic acids were standards for each plate. The reaction was monitored using the fluorescent dye dichlorolfluorescein. Peroxyl

radicals generated by 2,2'-Azobis(amidinopropane) (ABAP) oxidize nonfluorescent dichlorofluorescein (DCFH) to fluorescent dichlorofluorescien (DCF). The degree of inhibition of DCFH oxidation by AO that scavenge peroxyl radicals was used as the basis for calculating the AO activity. The reaction mix contained 0.75 M phosphate buffer at pH 7.4, 50 mM ABAP, 12.5 *u*M DCFH dye, and the appropriate concentrations of the sample extracts. The dye was prehydrolyzed with 1 mM KOH to remove the diacetate moiety just prior to use in the reaction, and the reaction was carried out at 37 °C, in a total volume of 200 *u*L using a 96-well plate. Fluorescence generation was monitored (excitation at 485 nm and emission at 538 nm) with a Fluoroskan Ascent fluorescent spectrophotometer (Thermo Labsystems, Franklin, MA). Data were acquired with the Ascent Software, version 2.6 (Thermo Labsystems, Franklin, MA) running on a PC. The areas under the fluorescence reaction time kinetic curve (AUC) for both control and samples were integrated and used as the basis for calculating peroxyl radical scavenging capacity (PSC) using the equation:

$$\text{PSC unit} = 1 - (SA/CA)$$

where SA is AUC for the sample or standard dilution and CA is AUC for the control reaction. Compounds or extracts inhibiting the oxidation of DCFH produced smaller SA and higher PSC values. The parameter EC_{50} was defined as the dose required to cause a 50 percent inhibition (PSC value of 0.5) for each sample extract and was used as the basis for comparing different compounds or samples. Results obtained for sample extract AO activities were expressed as *u*mol of vitamin C equiv (VCE)/mL of milk or CMR ± SEM for triplicates.

Statistical Analysis

Data were statistically analyzed using the PROC MIXED procedure of SAS

9.2 (SAS Inst. Inc., Cary, NC). The effects of fat content, protein content and CMR on AO activity were independently assessed. Data are reported as least square means ± SEM; effects were considered significantly different when $P < 0.05$.

RESULTS AND DISCUSSION

The AO activity of defatted natural bovine milk (52.7 µmol VCE/mL) analyzed was from cows fed maize silage-based TMR, (Table 4.1). AO activity of milk may vary according to the diet of the cow and the processing of the milk (Zulueta et al., 2009).

While there was no effect of protein content on AO activity in the samples analyzed, others have reported the casein fraction to be the major contributor to the total AO capacity of whole milk (Zulueta et al., 2009). However, type of protein (soy) had a positive effect on AO activity in the case of CMR A (Table 4.1). This can likely be attributed to the isoflavones and cinnamic acid derivatives present in soy. Despite its contribution to AO activity, the use of soy proteins in CMR is not optimal due to the anti-nutritional factors in soybeans that reduce the digestibility of soy protein, especially in young calves (Akinyele and Harshbarger, 1983), and the differences in AA profile from that of milk protein, (Kanjanapruthipong, 1998).

Although not observed in this study, a potential negative impact of many fat sources in CMR is the addition of high levels of unsaturated fatty acids prone to oxidation, which can actually be a source of reactive oxygen species (Dibner et al., 2011). The unintentional addition of pro-oxidants via feed ingredients is an area worthy of careful monitoring, as justified by the Novus International Survey from 2000 to 2005 (Dibner et al., 2011). Novus reported that 40 to 50% of the fat sold for

Table 4.1 Antioxidant activity of bovine milk and calf milk replacers[1]

ID	Description	Protein Source	Animal Fat, %	Vegetable Fat, %	VCE, µmol	SEM
A	21% CP, 20% fat	50% milk, 50% soy	100	0	86.0[a]	1.9
Milk	Bovine milk; 29% CP, 29% fat	Milk	100	0	52.7[b]	1.9
B	Fatty acid supplement[2]; 22% CP, 20% fat	Milk	98.4	1.56	44.3[c]	1.9
C	20% CP, 20% fat	Milk	100	0	16.1[d]	2.4
D	Fatty acid supplement[2]; 28% CP, 18% fat	Milk	98.6	1.39	14.9[d]	1.9
E	28.5% CP, 15% fat	Milk	100	0	12.1[d]	1.9
F	5% plasma; 22% CP, 20% fat	animal	100	0	10.5[d]	1.9

[1]Results for total AO activity are expressed as µmol of vitamin C equivalent (VCE)/mL of milk or reconstituted milk replacer

[2]Fatty acid supplement represents a commercial fatty acid supplement of specific short, medium and long polyunsaturated fatty acids

[abcd]Means with different superscript differ, $P < 0.01$

use in animal feeds was unstable, with even higher percentages of instability observed in warmer months of the year (Dibner et al., 2011). Thus, while CMR analyzed in this study were shipped from the manufacturer directly after mixing and subsequently stored at -20°C until analysis, CMR used on farms will be more susceptible to the effects of storage conditions and feed ingredient quality on AO activity.

With the exception of CMR A, AO activity of bovine milk was significantly higher than that of CMR. The difference in AO activity of bovine milk may be due in part to its AA composition (Clausen et al., 2009) and its increased fat content (Chen et al., 2003), which can influence the concentration of carotenoids (Lindmark-Mansson and Akesson, 2000). Moreover, vitamin content of milk and CMR often differ. Hydrophilic compounds such as vitamin C and uric acid were the largest contributors to the total AO capacity of deproteinized milk according to Zulueta et al. (2009). While reports of ascorbic acid (vitamin C) concentration in natural bovine milk range from 5.9 to 14.0 mg/L, (Lindmark-Mansson and Akesson, 2000) many CMR do not formulate for ascorbic acid.

In addition, the fatty acid profile of whole milk differs significantly from that of most CMR, which typically lack short and medium chain fatty acids. Some CMR, such as B and D, have attempted to better match the fatty acid profile of milk through the addition of a commercial fatty acid supplement containing specific short, medium and long polyunsaturated fatty acids. CMR B and D had different AO activity ($P < 0.01$) when the comparison was made based on the concentration of 150 g CMR/L of water. However, at this concentration, CMR D had roughly half the amount of vitamin and trace mineral premix (and therefore, half the levels of vitamins A, C, D and E) because the feeding recommendation is 1.02 kg/d, whereas CMR B is

formulated to be fed at 0.57 kg/d. If the comparison of AO activity had been made based upon total AO fed rather than concentration, then CMR B and D may not have had a significant difference in AO activity. Only one other CMR (E) had this increased feeding recommendation, as is customary for higher protein CMR designed for accelerated growth; all others were formulated to be fed at 0.57 kg/d. Therefore, the AO activity of CMR B (and likely D if the evaluation was made based on total AO fed), though still lower than that of milk, was higher than the other CMR, possibly due to this fatty acid supplement.

Although fat content tended to have an effect ($P = 0.057$) on AO activity in the samples analyzed, losses in lipid-bound AO are not fully accounted for because the milk and CMR were defatted prior to analysis. When milks with varying fat contents were analyzed for total AO activity, others reported a small contribution to total AO activity from the liposoluble AO components of whole milk versus skim milk (Zulueta et al., 2009). Future research is warranted to compare CMR with a broader range of fatty acid content and profiles using methods that account for total lipophilic and hydrophilic AO.

The AO activity of milk is the result of the combination of native antioxidative enzymes as well as non-enzymatic AO such as lactoferrin, vitamins C and E, phenolics and flavonoids, and fat-soluble carotenoids (Lindmark-Mansson and Akesson, 2000). In CMR, the AO activity is dependent upon the inclusion of non-enzymatic AO supplied as essential nutrients, most commonly vitamin E and selenium, and occasionally vitamin C.

CONCLUSIONS

Overall, opportunity exists to enhance AO activity of CMR to more closely mimic the AO activity of bovine milk. Despite exhibiting the highest total AO activity, CMR with a high percent of soy protein are generally not recommended due to the lower digestibility of soy protein. Natural bovine milk had the second highest AO activity. Fat, vitamin and mineral content, enzymatic AO, phenolics and flavonoids, fatty acid profile and AA composition are among the many factors that may explain the difference in AO activity between natural milk and formulated CMR. When comparing AO activity of CMR, it is important to consider the diversity in feeding recommendations, which will alter the amount of vitamin and mineral content, thus influencing AO activity. Strategies for increasing the AO activity of CMR should include ensuring the usage of high quality ingredients, proper storage of CMR, and the inclusion of dietary AO as essential nutrients.

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CHAPTER FIVE

PREDICTORS OF PERFORMANCE IN AN ANIMAL NUTRITION CLASSROOM

ABSTRACT

Animal Nutrition is a required course in animal science curriculums nationwide. Typical of required courses, students in the class are diverse in previous academic and animal experience. The objective of this research was to gather information about accurate student performance predictors to improve advising and course design. Data from 443 students, representing four semesters (Fall 2007-2010) of Animal Nutrition students, were statistically analyzed to determine predictive relationships between Scholastic Aptitude Test (SAT) scores, residency, transfer status, animal experience, major, gender, grade in a recommended Cornell general chemistry prerequisite (Cornell Chem) and performance in an introductory animal nutrition course (Animal Nutrition; Cornell University). In addition, an optional survey was administered to the 2010 Animal Nutrition class. In the survey, 27% of students self-identified animal experience level and 48% of transfers identified transfer status as influencers of their grade. Transfer status, residency, SAT scores, gender and grade in Cornell Chem were identified as significant predictors of performance in Animal Nutrition. The highest correlation for a predictor was gender followed closely by Cornell Chem grade; completion of Chem was associated with significantly higher Animal Nutrition grades. Gaining information about accurate student performance predictors can assist advisors in making course recommendations as well as instructors in designing the course to best enable learning regardless of the diversity in student preparation.

INTRODUCTION

Classroom diversity can manifest itself in a variety of ways. A heightened awareness of multicultural and ethnic diversity influences most university admissions policies as well as scholarship and extra-curricular programs. In animal science departments, the diversity of the undergraduate classroom has evolved considerably over the last century. It has been characterized by an increase in the proportion of women as undergraduates over the last 50 years, which is now approximately 50 to 75% of animal science majors (Beck and Swanson, 2003). At the same time, increases in racial diversity have been less dramatic, with Blacks and Hispanics still barely recognizable statistics at all levels of academic degrees conferred (Beck and Swanson, 2003). The focal point of this research, however, is to examine additional discipline-specific aspects of diversity, which have been observed as first emerging, and now growing, trends in animal science departments: lack of animal experience and increase in transfer students (Allen, 1983; Taylor and Kauffman, 1983; Buchanan, 2008).

Diversity in background animal experiences is often the result of increasing proportions of urban students, fewer students coming from or planning to return to family farms, and more students studying animal science with the intention of applying to a college of veterinary medicine (Buchanan, 2008) or other professional program. According to the 1910 U.S. census, when many animal science departments were first created, 33% of the U.S. population was engaged in farming and ranching as opposed to 1% of the U.S. population in the 2000 U.S. census (Britt et al., 2008). Thus, early teacher-scientists in animal science discussed the challenges of how to stimulate students to be ‘scholars as well as stockmen’ (Taylor and Kauffman, 1983). Now, many students who enroll in animal science have experienced animals solely as

companions rather than livestock (Britt et al., 2008). Moreover, the increasing number of students who transfer from a 2-year college (Buchanan, 2008) or after any combination of one to three years of post-high school study, has also increased classroom diversity in the form of academic preparation for the course and prerequisites taken. In addition, study skills developed for classroom success and the type of examinations given vary among schools, providing yet another area of adjustment for transfer students. Cherney and Bell (2002) also reported that transfer students in their animal nutrition course were less likely to indicate veterinary medicine as a vocational objective. In a department such as animal science where a large proportion of undergraduates plan to attain a post-graduate degree, this difference in postgraduate plans can further contribute to the diversity of student needs and expectations present in the classroom.

Obtaining information about accurate student performance predictors can assist advisors in making course recommendations; it can also aid instructors in designing the course to best enable learning regardless of the diversity in animal experience and course preparation. Thus, the objective of this research was to discover predictive relationships between SAT scores, residency, transfer status, animal experience, major, gender, grade in a recommended general Cornell chemistry prerequisite (Cornell Chem) and performance in an animal nutrition course.

MATERIALS AND METHODS

Animal Science Nutrition 2120 (Animal Nutrition) at Cornell University was chosen as a representative required course because the course material is a universal component of animal science curriculums nationwide. Moreover, there was no change

in the instructor or the major course material covered in the four years during which data was collected. Students enrolled in the course enter with a wide range of academic and animal experience due in part to the high rate of incoming transfer students. Transfer students made up 30% of the fall 2010 incoming animal science majors. Moreover, the large class size, averaging 110 students, poses a challenge for the instructor in relating information to students according to their personal knowledge level and experience.

This study was deemed exempt by the Cornell Institutional Review Board. Non-identifying information, coded anonymously from the College of Agriculture and Life Sciences (CALS) was collected regarding the following predictors of class performance in Animal Nutrition: transfer status, SAT scores, NY state residency, gender, major, grade in Animal Nutrition and grade in Cornell Chem. Data was analyzed using Proc Mixed of SAS 9.2 (SAS Inst. Inc., Cary, NC). Due to missing information for transfer students, results are displayed as each predictor individually regressed to Animal Nutrition course grade.

To supplement the data analysis and attempt to measure animal experience level of students, an optional survey (Appendix I) was administered to the fall semester 2010 Animal Nutrition class with a 90.3% response rate (Figure 5.1). Students did not receive an incentive and participation was voluntary.

RESULTS AND DISCUSSION

The data collected from the CALS Registrar represented a total of 443 students from four semesters (fall 2007 through fall 2010). The dataset was predominantly made up of sophomores and juniors (89%). Most of the sophomores were four year

students (93%) while the majority of the juniors in the course (77%) were transfer students. The high rate of transfer students in the Animal Nutrition dataset (29% overall) is not unlike that of other animal science programs with transfer students making up 19% of incoming animal science majors at the University of Wisconsin-Madison in fall of 2010, 29% at Texas A&M University and 22% at North Carolina State University (personal communication). In the optional survey administered to the fall 2010 Animal Nutrition course, 48% of transfer students self-identified transfer status as an influencer of their grade in the course (Figure 5.1). In response to the question of how transfer status influenced the respondent's Animal Nutrition grade, one student wrote, "Courses taken at other colleges were not as thorough as Cornell's equivalent classes" while another wrote, "Even though I'm a junior, this semester I feel like a freshman."

The mean grade in Animal Nutrition was 83.7 ± 8.8. Significant predictors of Animal Nutrition grade include SAT scores, (both math and verbal), grade in the Cornell Chem prerequisite, NY state residency, transfer status and gender. Results are summarized in Table 5.1. Students performed better in the course if they were non-residents of NY state (84.8 versus 82.5; $P = 0.007$), non-transfers (84.4 versus 81.3; $P = 0.001$), and female (85.0 versus 79.0; $P < 0.001$). Moreover, these three predictors were correlated with each other in that the majority of transfer students were male, NY residents. For each additional point received on the SAT Math score, there was an additional 0.043 point increase in Animal Nutrition grade; likewise a point increase in an SAT Verbal score was associated with a 0.039 point increase in Animal Nutrition grade. In a similar analysis at Oklahoma State University, Vitale et al. (2010) identified cumulative GPA, major, gender and performance in prerequisites to be

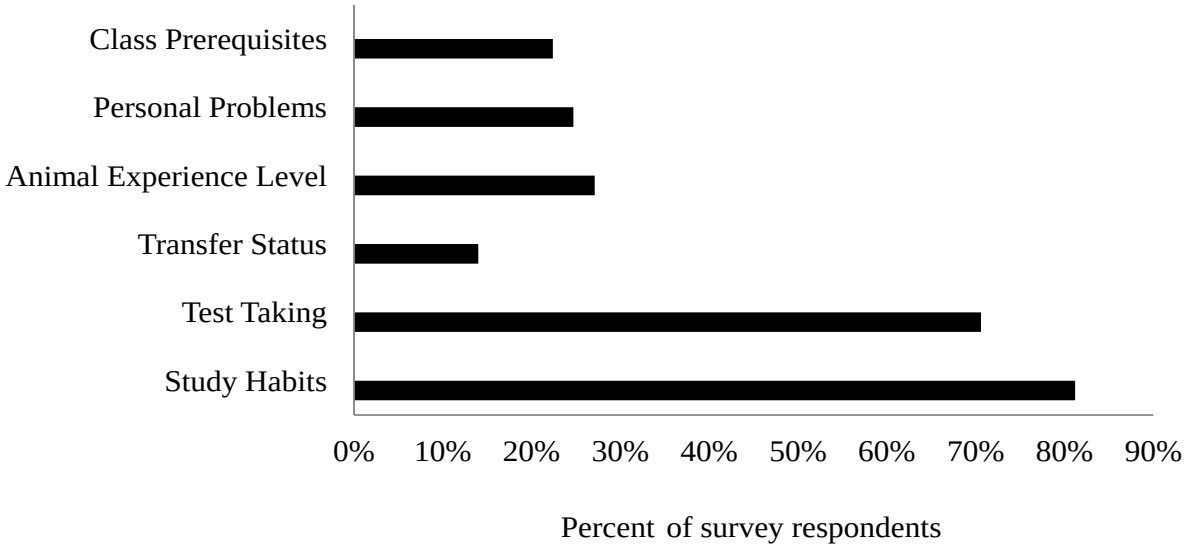

Figure 5.1 Student-identified influencers of performance in Animal Nutrition 2120, Cornell University.

significant predictors of student performance in an undergraduate agricultural economics classroom while race, residency, transfer status and high school GPA were not.

In a model that included the terms transfer status, NY residency, GPA, gender, SAT scores and major, 70% of the variation in Animal Nutrition grade was explained by these predictors. However, only 297 of 443 observations were included because any record with missing data was deleted from the analysis; the majority of deleted records were transfer students missing SAT scores. Although SAT scores were correlated with performance ($P < 0.001$), they were not on record for 87% of transfer students. This prevents definitive conclusions about the strength of the relationship between SAT score as a predictor of Animal Nutrition grade for transfers. Most universities post demographic information in a document called a Common Data Set, published by their Office of Institutional Research. In a survey of U.S. universities with strong animal science programs, the Common Data Sets reveal that the majority of universities report the use of standardized test scores for admissions of first year, first time students as *Very Important* or *Important*. However, the same report reveals that few if any of these institutions require standardized test scores for admissions of transfer students.

Moreover, transfer students rarely take the recommended general chemistry prerequisite at Cornell University, despite the fact that Cornell Chem grade had the second highest correlation ($r^2 = 0.409$) with Animal Nutrition grade of any analyzed predictor (Table 5.1). Students who simply completed the Cornell Chem received a significantly higher grade in Nutrition (86.3 versus 80.6; $P < 0.001$). Animal Nutrition has long been recognized as an applied science in which a background in the

fundamental sciences is ideal (Mitchell, 1936; Hoefer, 1968) and a strong foundational knowledge of chemistry appears to continue to significantly impact student performance in Animal Nutrition.

Cumulative GPA exhibited a high correlation but cannot be considered a true predictor of Animal Nutrition grade because GPA values were only present for students who had graduated Cornell University by fall 2010; thus grade in Animal Nutrition is a predictor of final cumulative GPA rather than the reverse. High school GPA was not available in the Cornell dataset used for this analysis, but others have observed that high school GPA can explain more of the variance in university degree completion than ACT score, high school rank or learning style (Garton and Kitchel, 2005).

Though it is difficult to measure the effect of animal experience level on student performance in Animal Science courses, it has been recognized as an important aspect of diversity in Animal Science undergraduate populations (Buchanan, 2008; Allen, 1983). Twenty-seven percent of students surveyed in the 2010 class felt animal experience level influenced their grade (Figure 5.1). In one question of the survey, students characterized the type of animal experience they held. The majority (87%) had experience with a pet whereas only 55% claimed actual farm experience. It is important to also note that farm experience included horse farms so the number of students with production livestock experience was actually less than 55%. Student comments on the survey included “I felt at a disadvantage because so many other people had so much experience already” and “Owning a pet did not help.”

To assist in alleviating student concern over lack of animal experience, the role of laboratory sections in providing practical animal experience has been and will

Table 5.1 Predictors of student performance in Animal Nutrition 2120 at Cornell University

Item		N	Grade	SE	*P* value	r^2
GPA[1]		174	12.3	1.04	$P < 0.001$	0.450
SAT[1] Math		299	0.043	0.006	$P < 0.001$	0.144
SAT[1] Verbal		299	0.039	0.005	$P < 0.001$	0.152
Chemistry[1]		227	0.543	0.044	$P < 0.001$	0.409
Chemistry	Yes	227	86.3	0.567	$P<0.001$	NA
	No	216	80.6			
Residency	Resident	251	82.5	8.73	$P = 0.007$	NA
	Non-resident	192	84.8			
Transfer status	Four year	314	84.4	0.765	$P = 0.001$	NA
	Transfer	129	81.3			
Major	Animal Sci	403	83.5	1.39	$P = 0.854$	NA
	Non-major	40	83.8			
Gender	Male	107	79.0	0.814	$P < 0.001$	NA
	Female	336	85.0			

[1]*Grade* signifies the additional point increase in Nutrition grade a student would expect to receive for each additional predictor point for these continuous variables.

remain a key component of animal science programs (Horvath and Inskeep, 1968). It is not possible to ascertain from this study whether student concern over their lack of animal experience as an influencer of their Animal Nutrition grade was real or perceived. In the future, pairing students according to their animal experience level in laboratories may be an effective strategy for handling this aspect of classroom diversity. Moreover, to address the needs of students who lack livestock animal experience, curriculum should continue to strive towards including fundamentals of livestock production as well as a variety of directed practical experiences. For example, Bell and Cherney (1999) integrated a semester-long lamb feeding trial into the Animal Nutrition course that allowed students to gain experience in handling and care of farm animals while applying theoretical lecture material.

Moreover, animal experience ranked third (27%) in student-identified grade influencers, considerably behind 'test taking' and 'study habits,' which affected 71 and 81% of students, respectively (Figure 5.1). Additionally, under "Other influencers of your grade in Animal Nutrition this semester" many students listed 'competing courses' (Figure 5.1). The self-identified issues of test taking and study habits were commonly shared among three-quarters of the class, and merit recognition by advisors and instructors. In order to address these student needs, instructors and advisors can utilize and promote local university resources, such as the Learning Strategies Center at Cornell University, and the optional one credit courses that many CALS programs offer to assist new students in adjusting to university life.

CONCLUSIONS

Transfer status is negatively associated with student performance in Cornell

University Animal Nutrition 2120. However, this research identified strategies that could be effective in improving the performance of transfers, such as utilizing SAT scores during advising or emphasizing course pre-requisites like Cornell University Chemistry. Although it was unclear from this study if student animal experience is influencing performance in Animal Nutrition, it has been recognized as an important aspect of diversity in animal science undergraduate populations. Pairing students based on animal experiences during laboratories may be an effective strategy at easing student anxiety due to lack of animal experience. Moreover, to address the needs of students who lack livestock animal experience, curriculum should continue to strive towards including fundamentals of livestock production as well as a variety of directed practical experiences.

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CHAPTER SIX

SUMMARY

As discussed in the literature review of chapter one, FA is a phenolic compound of interest due to its antioxidant, anti-cancer and antimicrobial properties as well as its ubiquitous nature in forages. However, much of FA is bound within the lignin complex of plant cell walls, unavailable for digestion and subsequent absorption when the plant is consumed. When enzymatic pretreatments are utilized in forage feeds, an increased amount of FA may be potentially released into a free form in the feed. Increased amounts of free FA in the diet of lactating dairy cows could have a variety of implications for both the animal as well as humans that consume the milk produced.

The likelihood of free FA transfer from oral dosage to plasma and urine versus feces is the topic of investigation in the second chapter. The study, conducted in ram lambs, also assessed the impact of different levels of free FA on the feed intake of the ram lambs. To consider the effects of similar dosage levels of free FA on rumen microbial NDF digestion, an accompanying in vitro experiment was conducted. As a result of these studies, we concluded that free FA was excreted in the urine as opposed to the feces, indicating its uptake via the plasma. At 3g, 6g and 9g dosages for 30 kg ram lambs, free FA did not inhibit lamb DMI when compared to the DMI of lambs during control periods. Moreover, there was no evidence of inhibition of microbial NDF digestion of the alfalfa fed to the ram lambs in the in vitro experiment.

Thus, the question of free FA transfer into milk of lactating dairy cattle was pursued in the third chapter. This research again confirmed that excretion of an oral dosage of free FA was primarily via the urine as opposed to the feces. In addition, there was a significant increase in the amount of free FA present in the milk of dairy cattle administered 150 g of free FA via bolus or rumen fistula. The discussion

explored the implications of increased free FA in milk for milk flavor and quality. This chapter also presented a novel method for quantifying free FA in bovine milk; only one other method has been previously reported in scientific literature to date. Although FA is a potent AO, it is unlikely that the amounts that could potentially be released from a dairy cow's diet would be enough to make milk a significant contributor to total AO consumed in the human diet. However, given its antimicrobial properties, future research would be of value to investigate the effects of increased FA in milk upon the shelf life and stability of milk and milk products made from milk with increased FA concentrations. It is also likely that milk with increased FA will have increased FA degradation products such as 4-vinylguaiacol and hippuric acid; the quantification of these compounds as a result of increased dietary free FA in lactating cows would be beneficial in determining potential sensory impacts on milk and milk products.

The benefit of studying one compound's contribution to total AO activity in milk is limited due to the potential effects of multiple compounds and their potential synergies. Thus, in the fourth chapter, we utilized the milk sample preparation procedure developed in the preceding chapter to prepare milk and calf milk replacer samples for assessment of total AO activities. A survey of milk replacers that varied in fat and protein source and content was conducted; their values of AO activity were compared with the AO activity of bovine milk. Five of the six milk replacers had AO activities significantly lower than that of milk, revealing potential opportunities to improve calf milk replacers through the inclusion of dietary AO. This could better fortify the calf in a stage of life where disease challenges are often high, and the calf's immune system could benefit from dietary AO. In addition, future research is warranted to compare calf milk replacers with a broader range of fatty acid content and profiles using methods that account for total lipophilic and hydrophilic AO.

The fifth and final chapter of this dissertation is the result of research in the scholarship of teaching and learning, conducted as part of the candidate's requirement for completion of the Cornell Center for Teaching Excellence's Future Faculty Teaching Certificate program. During her graduate program, as part of a teaching assistantship, the candidate taught as a teaching assistant in six different animal science courses for a total of eight semesters, co-instructed a summer course and then became the primary instructor for a summer course in Animal Husbandry the following year. A research project conducted in an animal nutrition classroom is reflected in this chapter, which has been accepted for publication in a journal for agricultural teachers across North America.

APPENDIX I

IDENTIFYING PREDICTORS OF STUDENT PERFORMANCE IN AN UNDERGRADUATE ANIMAL NUTRITION CLASSROOM SURVEY

1. What year represents your year of study?

   a. Freshman b. Sophomore c. Junior d. Senior

   Other_____

2. In what year of study did you begin studying at Cornell University?

   a. Freshman b. Sophomore c. Junior d. Senior

3. If you are a transfer student, where did you transfer from?

_____

4. If you are a transfer student, what was your GPA before you transferred into Cornell?

_____

5. What prerequisites to Animal Science 2120 did you take at Cornell University?

   a. Chemistry 207 _____ Grade Received _____

   b. Chemistry 208 _____ Grade Received _____

   c. BIO 102 _____ Grade Received_____

   d. BIO 103 _____ Grade Received_____

   e. Animal Science Course _____ Grade_____

   f. Animal Science Course _____ Grade_____

   g. Animal Science Course _____ Grade_____

6. How have these prerequisites influenced your preparation for the An Sci 2120 course?

7. What types of animal experience did you have prior to taking An Sci 2120? Circle all that apply and indicate the number of years of that type of experience adjacent.

    a. No animal experience

    b. Owning and caring for personal pets _____

    c. Farm experience _____

    d. Work experience _____

    e. Zoo experience _____

    f. Classes involving animals _____

    g. Other _____ _____

8. How has your animal experience influenced your preparation for the An Sci 2120 course?

9. Circle the range that best describes your University GPA prior to taking AnSc 2120:

4.0 3.5-3.99 3.0-3.49 2.5-2.99 2.0-2.49 <2.0

10. Circle the approximate grade you expect to receive in An Sc 2120:

A+ A A- B+ B B- C+ C C- D+ D below D

11. What factors affected your grade in An Sc 2120 this semester? Circle all that apply.

    a. Class prerequisites

    b. Study habits

    c. Amount of animal experience

    d. Test-taking

    e. Transfer status

    f. Personal problems

    g. Other _____

12. Would you be willing to share an example of how the above helped or hindered your success in this course?

13. What specific changes would you recommend that might improve the An Sc 2120 course in future semesters?